

THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Engineering Lipid Metabolism for Production of Oleochemicals  
in *Saccharomyces cerevisiae*

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CHALMERS UNIVERSITY OF TECHNOLOGY

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Cover:

Schematic illustration of the process through which cell factories for production of oleochemicals are generated. The pictures shows an crosstalk between scientific knowledge and application. The yeast cell is a fluorescent microscopy picture of fluorescent proteins localized to the membrane that surrounds lipid droplets.

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# Engineering Lipid Metabolism for Production of Oleochemicals in *Saccharomyces cerevisiae*

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## Abstract

Oleochemicals are chemicals usually derived from plant oils or animal fat. Large use of plant oil derivatives as replacements for petroleum-derived chemicals brings sustainability issues from extensive cultivation of oil plants in restricted regions. This project studied and developed the baker's yeast *Saccharomyces cerevisiae* as a platform for sustainable production of oleochemical precursors.

The first part of this work studied the dynamics of free fatty acids (FFAs) production. First, an alternative fatty acid synthesis system based on the reverse  $\beta$ -oxidation pathway was evaluated for its *in vivo* function but concluding that it was not an efficient route for fatty acid synthesis. The subsequent studies were based on high level production of FFA and secretion to the extracellular medium through removal of acyl-CoA synthase activity by deleting the *FAA1-4* genes. This phenotype was coupled to a pathway that converts FFA to fatty alcohols, which allowed the observation that while FFA are more efficiently converted to fatty alcohols during growth on glucose, the production of FFA is highly increased during growth on ethanol. Fine-tuning of *FAA1* expression resulted in improved production of fatty alcohols without FFA secretion in this strain. Following up, the pathways leading to FFA formation in a  $\Delta faa1 \Delta faa4$  background were studied through construction of a strain with a constrained lipid metabolism network. It was observed that upon removing storage lipid formation, phospholipid synthesis had a strong correlation with FFA production and FFA formation was mostly derived from phospholipid hydrolysis.

On the second part of this work, *S. cerevisiae* was engineered for the highest TAG production levels reported so far. This relied on overexpressing genes involved in malonyl-CoA supply and TAG synthesis from acyl-CoA, and removing genes involved in TAG hydrolysis,  $\beta$ -oxidation and glycerol-3-phosphate usage. On a second approach, TAG accumulation properties were further improved in these strains through enhancing lipid droplet assembly processes. This was achieved through expression of perilipins and FIT proteins and through stimulation of ER stress mechanisms.

In conclusion, lipid metabolism is an important part of cell homeostasis and engineering this system requires overcoming its tight regulation networks and mastering the processes involved in the physical structural organization of the system. Here this was highlighted using both knowledge-driven studies and engineering approaches, leading to important advancements in the field.

**Keywords:** *Saccharomyces cerevisiae*; lipid metabolism; oleochemicals; lipid homeostasis



# List of publications

This thesis is based on the following publications and manuscripts:

- I. Teixeira PG, Siewers V, Nielsen J. **Quantitative *in vivo* evaluation of the reversed  $\beta$ -oxidation pathway for fatty acid production in *Saccharomyces cerevisiae*.** *Manuscript, available at bioRxiv* (<https://doi.org/10.1101/201616>)
- II. Teixeira PG, Ferreira R, Zhou YJ, Siewers V, Nielsen J. **Dynamic regulation of fatty acid pools for improved production of fatty alcohols in *Saccharomyces cerevisiae*.** *Microbial Cell Factories* 2017;16: 45. (<https://doi.org/10.1186/s12934-017-0663-3>)
- III. Ferreira R\*, Teixeira PG\*, Siewers V, Nielsen J. **Redirection of lipid flux towards phospholipids in yeast increases fatty acid turnover and secretion.** *Proceedings of the National Academy of Sciences of the USA* 2018, *accepted*
- IV. Ferreira R, Teixeira PG, Gossing M, David F, Siewers V, Nielsen J. **Metabolic engineering of *Saccharomyces cerevisiae* for overproduction of triacylglycerols.** *Submitted Manuscript*
- V. Teixeira PG, David F, Siewers V, Nielsen J. **Engineering lipid droplet structural features for increased triacylglycerol accumulation in *Saccharomyces cerevisiae*.** *Submitted Manuscript*

\* authors contributed equally

# Contribution summary

**Paper I:**

Designed most of the study, performed all the experimental work, analyzed data and wrote the manuscript

**Paper II:**

Partially designed the study, performed most of the experimental work, analyzed data and wrote the manuscript

**Paper III:**

Performed about half of the experimental work (more on lipid analytics and less on strain construction), analyzed data and wrote the manuscript together with first co-author

**Paper IV:**

Performed some of the lipid analysis experiments, took part in writing the manuscript

**Paper V:**

Designed the study, performed all the experimental work, analyzed data and wrote the manuscript

# Preface

This dissertation is submitted for the partial fulfillment of the degree of doctor of philosophy. It is based on work carried out between August 2013 and November 2017 in the Division for Systems and Synthetic Biology at the Department of Biology and Biological Engineering, Chalmers University of Technology under the supervision of Professor Jens Nielsen. The research was funded by the Swedish Foundation for Strategic Research in the scope of a collaboration project with the KTH Royal Institute of Technology in Stockholm and the Center for Biosustainability (CFB) in Copenhagen. The project funding was aided by the Knut and Alice Wallenberg Foundation.

Paulo Gonçalves Teixeira

January 2018

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# Abbreviations

CL: Cardiolipin

DCW: Dry Cell Weight

ER: Endoplasmic Reticulum

FAS: Fatty Acid Synthase

FIT: Fat-Inducing Transmembrane Protein

FFA: Free Fatty Acid

LD: Lipid Droplet

PA: Phosphatidic Acid

PC: Phosphatidylcholine

PE: Phosphatidylethanolamine

PI: Phosphatidylinositol

PS: Phosphatidylserine

SE: Sterol Ester

TAG: Triacylglycerol

“If you thought that science was certain,  
well, that is just an error on your part”  
— Richard Feynman



# Prologue

## Metabolic engineering in a global context

In a world of sustainable development, all materials, chemicals and energy sources need to have a renewable life cycle. An environmentally negative effect of the life cycle of any product necessary for human development ties the growth of humanity to the deterioration of the ecosystems we live in. Consequently, this causes a decay in life quality, economic instability and geopolitical problems related to limited resource availability (Sanborn Scott, 2005). The use of fossil sources for energy and materials has therefore been a concern raised many years ago and the finding of real sustainable alternatives to replace these sources is paramount.

At current state of development, the dependency we have on fossil-derived production of fuels and chemicals is one of the major challenges for a sustainable development. Fossil energy sources are formed by natural processes, such as anaerobic decomposition of ancient million-years-old organisms that contain energy and carbon stored at the time of death of the ancient organism. Usage of these energy and carbon sources originates two main issues. Firstly, fossil sources are a finite resource, since generation of these takes millions of years, while they can be processed and readily used in a short time scale (from some minutes to a few years). Secondly, use of fossil sources releases carbon to the atmosphere that is not consumed at the same rate that it is released. Carbon emissions are one of the main causes of global warming and climate change (Crowley, 2000), which was featured as one of the 10 biggest global challenges in 2016 by the World Economic Forum.

As to the origin of this issue, the current dependence on fossil sources was created from an economic development due to historical events rather than from technical limitations regarding use of renewable sources. Since oil was found as a resource that could be extracted from the ground and provide a cheap and easy way to obtain energy and materials, much of the industry, technical production processes, consumer products and supply chains were developed optimally for the use of this resource. The development was exponentially stimulated through the developments of the industrial revolution in the 1870s and the two occurring World Wars that demanded a higher and faster supply of energy (Andres et al., 1999).

As much as efforts are being made to replace fuels and chemicals from non-renewable sources, viable solutions for an independence from fossil resources are not yet achieved, and some

industries are very far away from an optimally sustainable solution. Potential solutions need to offer not only a reliable technical feasibility, but also a cost-effective production process and to some degree have a level of integration with either currently existing industrial processes or consumer products in order to penetrate the existent and well-established production and supply chains. In short, development of technology to progress towards sustainable supply of energy and materials must necessarily comply with the 3 main pillars of sustainability: The technology needs to be environmentally sustainable by relying on renewable sources and being carbon neutral, i.e. the capture of atmospheric carbon during the production process should be equal or superior to the carbon emissions during usage. It also needs to be economically feasible in order to be compliant with a society's economic growth. Furthermore, opportunity for profit will stimulate a market involvement, creating therefore a driving force for corporate technology development efforts at a steady pace. As a last point, it needs to be socially beneficial and account for the impact on living communities, job market and impact on different social classes both on production and use of such alternative sources.

The work described here aims to provide progress and knowledge towards a sustainable solution for producing fuels and chemicals. By relying on the transformative power of the biochemical processes existent in nature, it is possible to create a variety of sustainable material and energy products using i) renewable feedstocks and ii) microbial cells capable of converting these into the desired products.

The following text aims to explore the potential of yeast cells as a catalyst for production of oleochemicals from fully sustainable sources. The first sections will focus on the molecular biology and biochemistry aspects behind it, offering background on the processes involved. The following sections will report on the scientific advancements to the field generated through the projects presented through this thesis, and their significance.

# Introduction to Oleochemicals

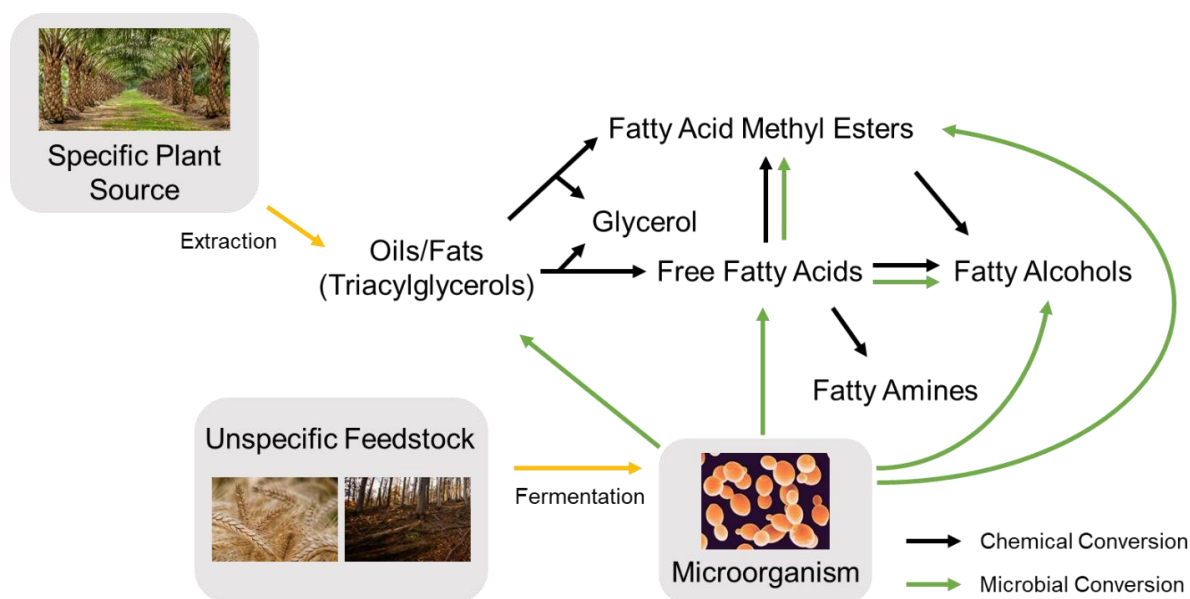
## Oleochemicals: Definition, processes and applications

Oleochemicals are defined as the group of chemicals that are extracted or derived from plant or animal fats (Rupilius and Ahmad, 2006). Because animal and plant fats are commonly composed of triacylglycerols, oleochemicals are characterized by the existence of one or more hydrophobic acyl-chains, which give them hydrophobic or amphipathic properties, depending on the product molecule.

Due to the presence of acyl chains, oleochemicals have developed into replacements to petrochemicals, which are chemicals derived from petroleum. One group of the most common petrochemical precursors are  $\alpha$ -olefins, or terminal alkenes, since the presence of a terminal carbon-carbon double bond allows for a series of different chemical reactions that transform this hydrocarbon into a chemical with a different terminal functional group, changing its properties and chemistry (Donohoe et al., 2009). In the same way, oleochemical processes use fatty acids (either in an unbound form or bound to a glycerol backbone) as a basic common precursor, in which the carboxyl group provides the needed reactivity for conversion into many other different functional groups.

Fats and oils present in animals and plants are mostly composed of triacylglycerols (TAGs) (Montero de Espinosa and Meier, 2011). TAGs are neutral lipid molecules with a glycerol backbone esterified to three fatty acids. A few exceptions exist to this, such is the case of jojoba oil, which is composed of wax esters (Miwa, 1971).

For oleochemical production, the TAGs from the fat material are the base material that through chemical reactions provides different basic industrial precursors (Metzger and Bornscheuer, 2006) which is schematically shown in **Figure 1**.



**Figure 1. Chemical routes for basic oleochemical production and possible microbial routes to replace them.** Traditional oleochemical industry typically extracts plant oils and chemically reacts them to generate other oleochemical species. Production of oleochemicals through microbial fermentation uses different biomass as feedstock. Through an engineered metabolism, the microorganism can produce any of the highlighted oleochemicals. (adapted from Pflieger et al., 2015)

The basic oleochemicals, which can be simply converted from fat material and serve as precursors to all other oleochemical products are: free fatty acids, fatty methyl esters, fatty alcohols, fatty amines and glycerol (Biermann et al., 2011; Pflieger et al., 2015; Salimon et al., 2012). Glycerol is the only one of these molecules that does not have an acyl chain and it is a byproduct of hydrolyzing the TAGs in fat into its single fatty acid constituents. Glycerol is a mostly unattractive product with a low market value and for that reason will be excluded from the definition here of oleochemical products of interest. The focus will instead be on oleochemicals with an acyl chain.

Some basic oleochemicals, like free fatty acids or fatty acid methyl esters, can be derived directly from TAG conversion while others, such as fatty alcohols and fatty amines, are derived from chemical conversion of other basic oleochemical species. Fatty alcohols can be produced either from free fatty acids or fatty methyl esters while fatty amines must be derived from free fatty acids (Salimon et al., 2012). The enumerated basic oleochemicals can then be converted into more advanced chemicals with different applications to a variety of products. A short summary of these derived chemicals and applications is presented in **Table 1**.



**Table 1. Examples of chemicals and applications derived from basic oleochemicals.**

Basic Oleochemical	Free Fatty Acids	Fatty Acid Methyl Esters	Fatty Amines	Fatty Alcohols
<b>Derivative Chemicals</b>	Fatty Acid Esters Conjugated Fatty Acids Alkyl Epoxyesters	Sulfo Fatty Acid Ester	Fatty Amine Oxides	Fatty Alcohol Sulfates Fatty Alcohol Ethoxylates Fatty Alcohol Ethoxysulfates
<b>Major Applications Examples</b>	Soaps & Detergents Chemical Intermediates Plastics Personal Care	Biodiesel Surfactants	Fabric softeners Cosmetics Corrosion Inhibitors Antimicrobials	Soaps & Detergents Personal Care Lubricants

## Oleochemicals: Raw materials, needs and sustainability

The most common sources of fats for production of modern oleochemicals are plant oils. Which oil to use depends on the properties of the desired final product in terms of the desired acyl chain composition. Fatty acid composition changes between different plants and different tissues in terms of fatty acid chain length and presence of desaturations, which ultimately affect the properties of the final product (Carlsson, 2009).

In 2017, the world production volume of vegetable oils reached 200 Million metric tons, from which at least 15% are estimated to be used for non-edible purposes (Rupilius and Ahmad, 2006; Tao, 2007). The largest sources of world vegetable oils are palm and soybean, which together account for 65% of the total world production of plant oils (<https://www.statista.com>).

Dependency on specific plant species for production of oleochemical precursors raises two main question. Firstly, the limited variety of fatty acid compositions in high-producing plants such as palm and soybean limits their use for applications where specific fatty acids are needed. In this case it is necessary to rely on more exotic plant species that can have low productivity or require cultivation under specific climate and soil conditions. This creates economic dependencies and supply chains controlled by few key players, which decreases flexibility of sources and increases instability of supply rates and prices. Secondly, both palm and soy require favourable climate and soil conditions to achieve a high productivity. As such, 85% of the world palm oil supply comes from Indonesia and Malaysia while 80% of soybean oil is

produced by the US, Argentina and Brazil (<http://faostat.fao.org/>). This monoculture of a single plant species only in a specific region and relying on those few regions to keep up with world demand leads to severe environmental problems such as deterioration of soils, deforestation and destruction of habitats (Danielsen et al., 2009) as well as social concerns such as land-grabbing and exploitation of small farmer workers by large players in the plant oil industry (Gellert, 2015).

Although the use of plant oils is a significant improvement in the sustainable and economic development of replacing petroleum-derived chemicals, it is by far not enough or optimal as the only solution if the aim is to achieve a fossil-fuel independent industry. There is a need to supply oleochemical products in which the production process can be: i) flexible in terms of raw material input and product output, in order to reduce dependency on localized production of specific oils and reduce transportation, ii) easily modifiable and specific in order to produce the desired products (specific chain lengths, unsaturation, position of functional groups) with a reduced amount of contaminants or undesired species and iii) sustainable, both economically, environmentally and socially.

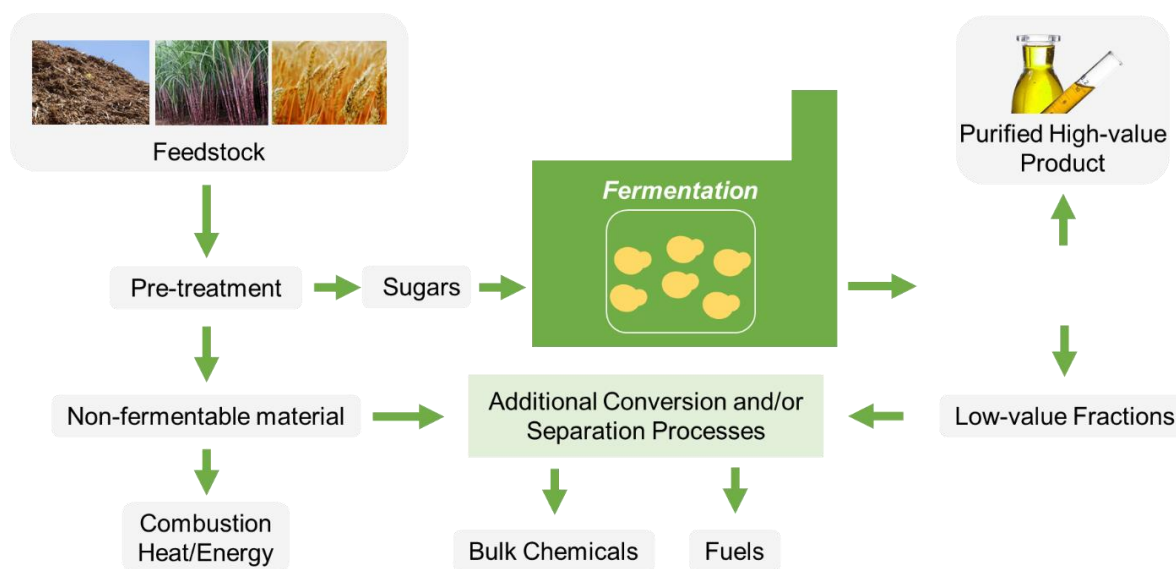
## Microbial-based production of oleochemicals based on engineered microbes

Microbes have been used in industry for a variety of purposes. Historically we see fermentation of food products as a prevalent theme throughout the ages with the advent of beer and wine brewing, bread baking, dairy processing and acetic conservation. Fermentation in this context can be simply described as cells consuming a carbon source, usually some type of sugar, and transforming it into another product such as ethanol or acids while harvesting energy from the process in order to grow. During fermentation, microbial cells are converting one product into another using a series of enzymes and reactions that are connected systematically and methodically and act as an assembly line. Conceptually, these cells are microbial cell factories and can be further developed and engineered for industrial conversion of a substrate into a specific product.

### Short introduction to the biorefinery concept

The biorefinery concept consists of a group of processes able to separate biomass resources such as corn, energy grass or wood residues into different fractions (sugars, lignin, hemicellulose) to be used in generating high-value products, biofuels and chemicals (Cherubini, 2010). Economic viability of a biorefinery relies on the production of at least one value-added chemical. This often requires the use of specialized microbes to catalyze this conversion of the feedstock into the specific desired products. Coupled to this is the processing

of the biomass (pre-treatment) to separate it into a part that can be available to be consumed by the microbial cells and another that can be used for lower value outcomes such as combustion for heat generation and lower value product production (Ragauskas et al., 2014). After the fermentation, downstream processing is needed to separate and purify the products resulting from the fermentation (Kumar and Murthy, 2011) (**Figure 2**), which will isolate high value products that are usually required in high purity from other bulk chemicals and fuels that can be used in low purities or separate to different phases (Kaparaju et al., 2009). This process can be coupled to other different chemical and biological routes, even involving other fermentation reactions, in order to have a sustainable and economically viable production chain.



**Figure 2. A simple biorefinery concept.** Low value biomass feedstock enters the process, from which the main goal is the production of value-added chemicals with generation of heat, energy, bulk chemicals and/or fuels in the process.

Microbial cells are the main player in a biorefinery focused towards specific high-value products and the choice of microbial cell will decide which feedstocks can be used as substrates, which products can be produced from this biomass, and have a heavy impact on production yields, titers and rates. Exactly for this reason, the viability of a biorefinery applications needs to rely heavily on the development of capable, efficient and robust microbial cells.

Microbial cells found in nature have been naturally evolved to maximize their growth and competitiveness in their native habitat, and as such their metabolism is adapted towards objectives different from the ones desired when using these in an industrial setting. Therefore, for industrial purposes, in order to specifically convert the feedstock into the desired product molecule and optimize the yield and rate of production of said molecule, cell metabolism needs

to be rewired and re-purposed through metabolic engineering (Keasling, 2010). The importance of metabolic engineering for a sustainable development namely for production of fuels and chemicals has been recognized by the World Economic Forum through listing the technology as one of the Top 10 Emerging Technologies of 2016.

## Potential and limitations of *Saccharomyces cerevisiae* for production of oleochemicals

### *S. cerevisiae* as an industrial organism

The baker's yeast *Saccharomyces cerevisiae* has been used by humans for thousands of years. Through the development of beer and wine brewing, as well as bread making, this yeast became an important organism in our food industry and consequently our society. Due to its incredible efficiency at fermenting sugars and producing ethanol, *S. cerevisiae* has also been used in the past decades for production of bioethanol as a fuel through fermentation of sugar sources such as sugarcane, corn, wheat and beetroot (Balat et al., 2008). Production of ethanol as an economically viable biofuel allowed for formulations including blending up to 15% in gasoline for regular automobile engines, extending to the development of engines running on fuels with higher percentage of this ingredient.

The parameters that allowed the success of *S. cerevisiae* for applications in industry are mostly its fast growth rate, robustness and resistance to low pH and fermentation inhibitors (Borodina and Nielsen, 2014; Cakar et al., 2012). This adds to the fact that it is generally regarded as safe (GRAS) organism to handle, proving no danger regarding pathogenicity, virulence or environmental hazards. Furthermore, the applications of this yeast in industry have led to extensive knowledge on its handling and working conditions in applied settings. Together with the existence of many applied techniques and procedures, this makes *S. cerevisiae* a favourite organism to handle in industrial applications (Krivoruchko and Nielsen, 2015).

Besides the consolidated knowledge on applications, *S. cerevisiae* has been intensively studied as a model organism for many eukaryotic molecular pathways due to the conserved nature of many of these with higher eukaryotes (Botstein et al., 1997; Petranovic et al., 2010). Due to this, *S. cerevisiae* is one of the most well studied eukaryotic organisms and benefits from vast knowledge, literature resources and extensive data deposited at numerous databases (Dolinski and Troyanskaya, 2015; Engel et al., 2014).

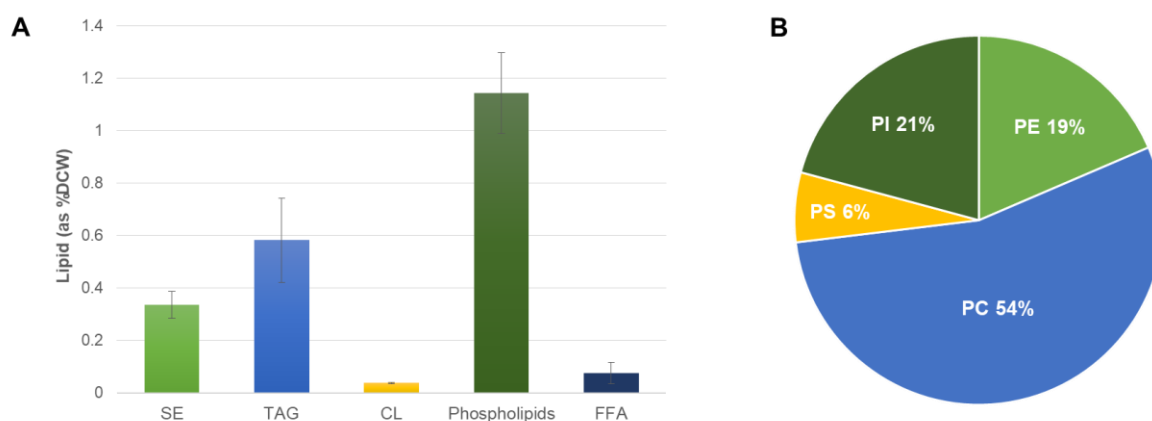
Through the years, the consolidated knowledge allowed the development of many tools for engineering this organism (Keasling, 2012; Murray et al., 2016). Early on, plasmid-based gene cloning and expression systems were set up, in which today the most commonly used are gene expression from 2 $\mu$  plasmids which can multiply in the cell to up to 80 copies allowing strong

expression of genes (Christianson et al., 1992), and centromeric (CEN) plasmids based on an ARS origin of replication. CEN.ARS plasmids replicate like chromosomes and usually only one single copy is kept in the nucleus (Rose et al., 1987). These are therefore important when a fine-tuned or controlled expression is desired. We also have a vast knowledge on gene promoter strength and inducibility conditions, and can therefore work with concepts of fine-tuned and dynamic gene expression by placing the genes of interest under control of specific promoters (Hubmann et al., 2014). Genome engineering such as gene knockout has also been shown to be a straightforward process in yeast, since homologous recombination is a very efficient DNA repair mechanism in this species, making it easy to specifically target a gene and replace it by a genetic marker that will then allow for selection of positive clones with the correct gene deleted (Nihei and Kishi, 2017). Through the same way, it is simple to integrate into the genome specific genes of interest to be expressed. These genes can be integrated with a genetic marker and selected in the same way. More recently, development of the CRISPR-Cas9 technology allowed for efficient genome engineering without the use of marker genes (Jensen et al., 2017; Mans et al., 2015). Due to high efficiency and selection by DNA repair capacity of the cell, CRISPR allowed for more time-efficient and simpler gene removal and insertion, speeding up the process of genome engineering. This efficiency and specificity in engineering, as well as the multitude of tools and knowledge available, make *S. cerevisiae* the preferred eukaryotic organism for precise cell factory development.

## Limitations of *S. cerevisiae* for production of oleochemicals

Even though this organism is exquisite when it comes to engineering potential, it natively has a poor capacity for production of oleochemical precursors. Our analysis of a typical *S. cerevisiae* strain shows that only 2-3% of its dry cell weight is composed of lipids, of which approximately 53% are phospholipids, 27% TAGs, 15% sterol esters (SEs) and 3% free fatty acids (FFAs) (**Figure 3A**).

Overlaying the lipid species produced by *S. cerevisiae* with potential molecules that are used as oleochemical precursors for industrial purposes highlights the potential of producing TAGs and free fatty acids using this organism. However, these two lipid species account for less than 1% of the total cell dry biomass, and are therefore natively present in too low amounts to be viable in terms of production processes. Other yeast species exist and have been investigated for their capacity to accumulate a high percentage of their biomass as lipids, specially TAGs. Yeast genera such as *Rhodospiridium*, *Rhodotorula*, *Yarrowia*, *Cryptococcus*, *Candida*, *Lipomyces* and *Trichosporon* are considered oleaginous since some of their species are able to accumulate more than 20% of their dry cell weight as lipids, reaching values as high as 70% (Adrio, 2017; Patel et al., 2016). While these species do offer a better starting point in cell factory development, they lack the engineering tools, knowledge and data available for *S. cerevisiae*, as mentioned above.



**Figure 3. Typical lipid composition of *S. cerevisiae* grown in minimal medium.** A) Quantification of the different lipid classes in *S. cerevisiae* displayed as percentage of dry cell weight. B) Distribution of the different phospholipid classes within the phospholipid fraction. SE: sterol esters; TAG: triacylglycerol; CL: cardiolipin; FFA: free fatty acids; PI: phosphatidylinositol; PE: phosphatidylethanolamine; PC: phosphatidylcholine; PS: phosphatidylserine. Data was obtained from a compilation of the datasets generated for the control strains during the studies in this thesis. Strains were cultivated for 72h in minimal media with 2% glucose

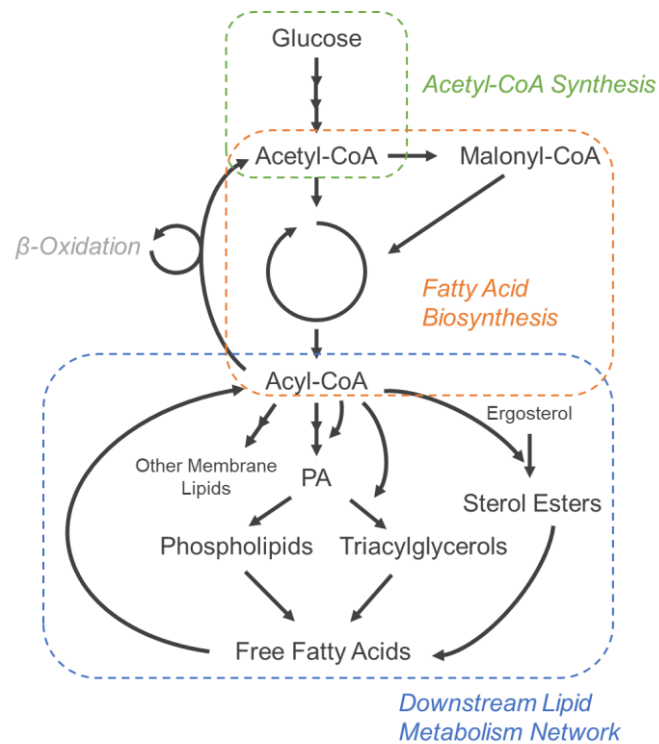
The factors described so far show a promising route towards using available engineering tools to develop *S. cerevisiae* cell factories for overproduction of either lipid species that can be used as oleochemical precursors, such as TAGs, or production of basic oleochemical species such as free fatty acids. This task, however, presents many challenges that will gradually be exposed in following sections. Overcoming these challenges requires a deep understanding of the lipid metabolism in this organism, as well as its overlaying regulation networks and the biological processes governing the organelles involved in these reactions. The following section will therefore focus on lipid metabolism in *S. cerevisiae* which will then allow us to expand this knowledge to design strategies for *in vivo* production of oleochemicals and its precursors.

# *Saccharomyces cerevisiae*: An Overview of Lipid Metabolism

## Lipid metabolism in *S. cerevisiae*

Lipid metabolism in *S. cerevisiae* involves an interplay between metabolic and regulatory networks (Jewett et al., 2013). These two networks communicate between each other to regulate levels of lipid pools and control the fatty acid composition of these (Henry et al., 2012). In **Figure 4**, the lipid metabolic network is presented in a simplified diagram. In terms of understanding of this network, a conceptual subdivision of all reactions is presented and here fatty acid biosynthesis is represented as the central process of lipid metabolism due to its importance. The fatty acid biosynthesis machinery produces acyl chains of up to 18 carbons based on 2 carbon monomers from acetyl-CoA. We can therefore divide lipid metabolism into 3 main parts: i) Production of acetyl-CoA as a substrate for fatty acid biosynthesis, ii) The fatty acid biosynthesis machinery, and iii) the transfer of acyl-chains in the form of acyl-CoA into other lipid forms, including the interconversion and transfer of acyl-CoA between different lipid classes.

The work developed here does not focus on engineering acetyl-CoA supply and only touches lightly on aspects of the fatty acid biosynthesis machinery, focusing mostly on the downstream lipid metabolism networks related to synthesis of different lipid classes from acyl-CoA, as well as acyl-CoA and fatty acid homeostasis and turnover through different lipids. For that matter, acetyl-CoA synthesis is here underrepresented and schematically simplified, since it is an extensive and complex subject on its own which would extend this thesis beyond its scope.



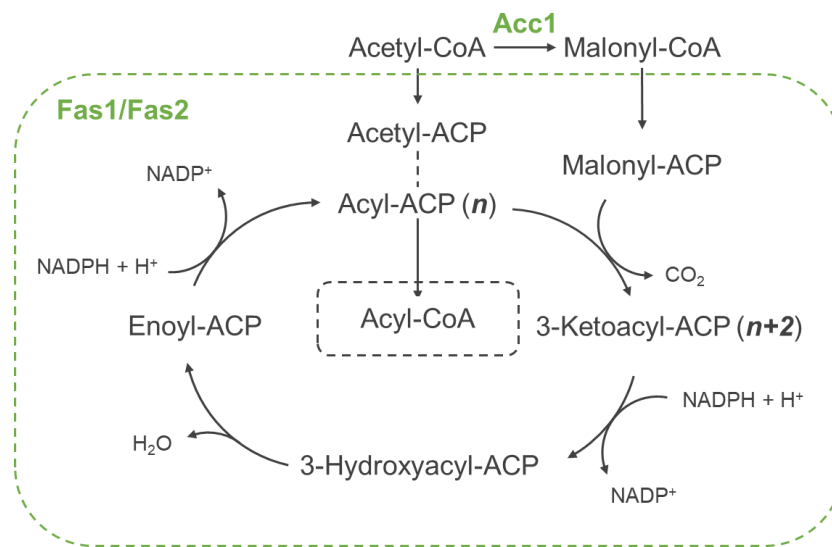
**Figure 4. Simplified representation of lipid metabolism in *S. cerevisiae*.** Cytosolic acetyl-CoA is produced from glucose or ethanol through complex pathways not displayed here. Cytosolic acetyl-CoA is then converted to malonyl-CoA, which is the building block of the fatty acid biosynthesis machinery. The product of fatty acid biosynthesis, acyl-CoA, is then used in a multitude of processes for production of membrane lipids such as phospholipids and neutral lipids such as TAGs and SEs. Phosphatidic acid (PA) is a common intermediate in the phospholipid and TAG synthesis pathways. Remodeling and hydrolysis of TAGs, phospholipids or SEs results in free fatty acids that are then re-activated to acyl-CoA, which can then re-enter lipid synthesis processes or be oxidized back to acetyl-CoA.

## The fatty acid biosynthesis machinery

Fatty acid biosynthesis entails a group of reactions that allow the condensation of multiple acetyl-CoA molecules in one acyl-CoA molecule, each acetyl-CoA contributing 2 carbon atoms to the acyl-CoA chain length (Tehlivets et al., 2007). This process happens in the cytosol and it is catalyzed by two distinct components: First, the acetyl-CoA carboxylase encoded by the *ACC1* gene catalyzes the conversion of acetyl-CoA to malonyl-CoA. The second component is the Fatty Acid Synthase (FAS) complex, a multifunctional enzymatic complex with seven different catalytic domains and one acyl carrier protein (ACP) domain. This complex is composed by two subunits  $\alpha$  and  $\beta$ , organized as a hexamer ( $\alpha_6\beta_6$ ) (Schweizer and Hofmann, 2004), each subunit encoded by the genes *FAS1* and *FAS2*, and catalyzes all the other reactions necessary for the synthesis of a fatty acyl-CoA molecule. The FAS complex uses acetyl-ACP as a primer for the first cycle and the extending acyl-ACP for the following cycles, consuming one malonyl-ACP and 2 NADPH during each cycle to extend the acetyl/acyl-ACP chain by 2 carbons through the reactions depicted in **Figure 5**. When the acyl-



ACP chain has reached a specific length, which in *S. cerevisiae* is often a 16 or 18 carbon chain, the acyl-ACP is converted to acyl-CoA by the MPT domain of the FAS complex and released to the cytosol where it becomes the substrate of other enzymes for further synthesis of other lipid species (Schüller et al., 1992; Schweizer and Hofmann, 2004; Tehlivets et al., 2007; Zhu et al., 2017). C16 or C18 fatty acids can be elongated to very long chain fatty acids of C20 to C26 through the action of an elongation system in the ER composed of the elongase enzymes Elo2, Elo2 and Elo3. The reaction involves cyclic steps analogous to the FAS system and also uses malonyl-CoA as a building block, but instead relying on -CoA intermediaries for the process (Aung et al., 2013; Tehlivets et al., 2007).



**Figure 5. Fatty acid biosynthesis through the yeast FAS complex.** Synthesis of fatty acids as acyl-CoA through the fatty acid synthase (FAS) complex. Malonyl-CoA is used as an elongation block and is synthesized by the acetyl-CoA carboxylase Acc1 from acetyl-CoA. The elongation reactions are done by the FAS complex which is a multifunctional enzyme complex generated by oligomerization of the polypeptides Fas1 and Fas2. Each cycle extends the size of the acyl-chain in the form of acyl-ACP by 2 carbons at the cost of 1 malonyl-CoA and 2 NADPH. Acyl-ACP molecules of usually 16-18 carbons are converted to acyl-CoA still by the FAS complex, which is after this released.

## The fates of fatty acyl-CoA

Acyl-CoA is a group of molecules having many fates. They are substrates of many different enzymes and their levels are a target of tightly controlled regulation. All this makes these molecules among the most important nodes in lipid metabolism networks.

Acyl-CoAs are substrates in pathways for synthesis of phospholipids (Carman and Han, 2011; Henry et al., 2012), TAGs (Liu et al., 2012), sterols esters (Bailey and Parks, 1975; Zweytick et al., 2000) and sphingolipids (Cowart and Obeid, 2007) and can take part in reactions

localized in a number of different organelles depending on its purpose (Henry et al., 2012): acyl-CoAs in the peroxisome will be targeted by the beta oxidation machinery to be oxidized back to acetyl-CoA to be used as an energy and carbon source by the cell (Poirier et al., 2006); cytosolic acyl-CoAs can be associated with phospholipid synthesis at the ER membrane (Carman and Henry, 1999) and with TAG synthesis in lipid droplets (LD) (Czabany et al., 2007). Acyl-CoA has been shown to be transported between organelle membranes and enzymatic processes bound to an acyl-CoA-binding protein (ACBP) (Rasmussen et al., 1994). This acyl-CoA-ACBP complex is also involved directly and indirectly in regulation of acyl-CoA levels as it is described in the following section (Knudsen et al., 1999).

Due to their importance and involvement in different metabolic processes, acyl-CoAs play a central role in lipid metabolism regulation. Their synthesis is feedback regulated through modulation of Acc1 activity, which catalyzes a limiting step in fatty acid biosynthesis (Brownsey et al., 2006).

When the acyl chains from neutral lipids or phospholipids are cleaved off as FFAs for remodeling or oxidation, FFAs are converted back to acyl-CoA in order to be reintegrated into lipid pools or oxidized in  $\beta$ -oxidation (Henry et al., 2012). The same happens when fatty acids are fed in the extracellular medium, where in order to be used in any way by the cell, these need to be activated to acyl-CoAs by the acyl-CoA synthases Faa1-4 or Fat1 (Black and DiRusso, 2007; Faergeman et al., 2001). The function of acyl-CoA synthases in the regulation of free fatty acid and acyl-CoA levels is explored in more detail in a later chapter.

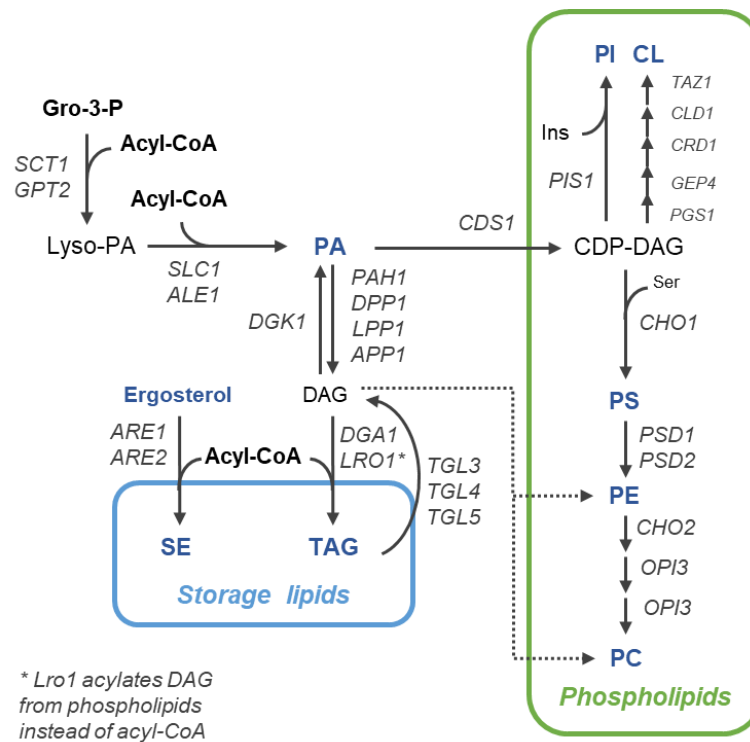
## Phospholipid biosynthesis

Phospholipids are the main components of cellular membranes (Gaspar et al., 2006; Spector and Yorek, 1985; Wagner and Paltauf, 1994). For this reason, these are probably the most important lipids with regard to cell viability, growth and correct function. Phospholipids are structurally composed of a phosphoglycerol backbone esterified with 2 acyl chains. Their synthesis starts with the esterification of a glycerol-3-phosphate with an acyl-CoA by the glycerol-3-phosphate acyltransferases Sct1 and Gpt2, forming Lysophosphatidic acid (LysoPA) and releasing the free CoA. A second step is the esterification of the LysoPA with a second acyl chain from another acyl-CoA by the lysophospholipid acyltransferases Slc1 and Ale1, forming PA (Carman and Henry, 1999; Chen et al., 2007). PA is an important branching point in lipid metabolism, and regarding phospholipid biosynthesis it is the common node between two different phospholipid biosynthesis pathways: the Kennedy pathway, and the CDP-DAG pathway (Carman and Han, 2011).

Through the Kennedy pathway, PA is first dephosphorylated by the phosphatidic acid phosphatase Pah1 to diacylglycerol (DAG) (Han et al., 2007). If ethanolamine or choline are supplied in the growth media, these are activated to CDP-ethanolamine and CDP-Choline, respectively, and through the action of the phosphotransferases Ept1 or Cpt1, these can react

with DAG to form either phosphatidylethanolamine (PE) or phosphatidylcholine (PC) (Gibellini and Smith, 2010). This pathway requires external supply of either ethanolamine or choline since *S. cerevisiae* is not able to synthesize these molecules *de novo*.

On the other hand, the CDP-DAG pathway enables synthesis of a variety of phospholipids using *de-novo* synthesis of all necessary components. Through this pathway, PA is first converted to CDP-DAG by the phosphatidate cytidylyltransferase Cds1 (Shen et al., 1996). CDP-DAG is then a branching point to different phospholipids. It can react with inositol by the action of Pis1 to form phosphatidylinositol (PI), it can form cardiolipin (CL) through a pathway composed of by Pgs1, Gep4 and Crd1, and it can be converted to phosphatidylserine (PS) by Cho1. PS can then be transformed into PE by Psd1 and Psd2, which can be further converted to PC by Cho2 and Opi3 (Henry et al., 2012) (**Figure 6**).



**Figure 6. Schematic representation of phospholipid and storage lipid synthetic pathways.** Phospholipid and triacylglycerol (TAG) synthesis pathways share a common initial pathway until PA (Phosphatidic acid) formation. PA can then be converted into CDP-DAG for formation of all the different phospholipid species or converted into DAG. DAG can still be converted into PE or PC if ethanolamine or choline are supplied to the medium. TAGs are produced through acylation of DAG through the enzymes Dga1 which acylated DAG from acyl-CoA or Lro1 which acylates DAG using a phospholipid acyl chain. PA: phosphatidic acid; DAG: diacylglycerol; SE: sterol esters; TAG: triacylglycerol; CL: cardiolipin; PI: phosphatidylinositol; PE: phosphatidylethanolamine; PC: phosphatidylcholine; PS: phosphatidylserine.

Due to their importance to cell vitality and function, phospholipid biosynthesis pathways have some redundancy as it is shown by the presence of two distinct pathways for formation of PE and PC, two of the most abundant phospholipid species in this yeast. Besides this, phospholipids can be remodeled through the action of phospholipases that cleave the acyl chains and acyltransferases capable of attaching new acyl chains (Merkel et al., 1999). This remodeling is important for maintaining membrane properties affected by the fatty acid species that compose the phospholipids (Schneiter et al., 1999; Spector and Yorek, 1985; Zinser et al., 1991). Furthermore, phospholipid biosynthesis is subject to regulation mechanisms that extend to many other aspects of lipid metabolism in order to maintain this important homeostasis and required balance for membrane stability (Chen et al., 2007; Lopes and Henry, 1991). The particular aspects of this metabolic regulation are presented further ahead.

## Storage lipid biosynthesis

Storage lipids are usually neutral lipids, such as TAGs and SEs. In rare cases, some organisms store fatty acids in other forms of neutral lipids, such as wax esters (Benson and Lee, 1972). Storage lipids are lipids that can be mobilized by the cell when needed. This involves oxidation of fatty acids for energetic purposes, i.e. using lipids as a source of energy and carbon such as seen in humans, who can store large amounts of TAGs in adipocytes than can be mobilized in periods of hunger (Horton et al., 1995). The other use of storage lipids is to store fatty acids that are not to be included in the membranes of the cell, either for being toxic (Listenberger et al., 2003; Plötz et al., 2016) or for not having the desired saturations/chain lengths required at the given time, and therefore are stored in TAGs until mobilized for remodeling of the phospholipid acyl chains (Renne et al., 2015).

Mutants of *S. cerevisiae* lacking synthesis of storage lipids are viable, and these lipids are not required for growth under normal culture conditions (Sandager et al., 2002). However, lack of neutral lipid synthesis causes an inability to respond to certain fatty acid toxicity. A case of this is oleate. When oleate is supplied in the media of a growing *S. cerevisiae* strain lacking storage lipid synthesis, it drastically increases in toxicity since the cell is not able to store it as a neutral lipid and therefore incorporates it excessively in its phospholipids, perturbing the stability of cell membranes and causing growth defects (Listenberger et al., 2003).

SEs are synthesized primarily by the acyl-CoA:sterol acyltransferases Are1 and Are2, which esterify the acyl chains from acyl-CoA with ergosterol molecules (Zweytick et al., 2000). These esters can be hydrolysed by the sterol ester hydrolases Tgl1, Yeh1 and Yeh2 providing release of ergosterol and fatty acids (Köffel et al., 2005).

TAGs are produced by acylating DAGs through the action of the diacylglycerol acyltransferase Dgal which uses DAG and acyl-CoA to synthesize TAGs (Sorger and Daum, 2002) or the lecithin cholesterol acyl transferase homologue Lro1, which synthesizes TAGs by transferring acyl groups from the *sn*-2 position of a phospholipid to diacylglycerol, thus forming an *sn*-1-

lysophospholipid and TAGs as products (Oelkers et al., 2000). Since TAGs are synthesized from DAGs, TAGs share part of their biosynthetic pathway with phospholipids. This is especially important because TAGs can quickly be remodeled to DAGs through the action of lipases such as Tgl3, Tgl4 and Tgl5 (Gaspar et al., 2011; Klein et al., 2016; Schmidt et al., 2013). These DAGs can either enter the Kennedy Pathway or be phosphorylated back to PA by the diacylglycerol kinase Dgk1 (Han et al., 2008a), allowing synthesis of phospholipids through the CDP-DAG pathway. There has been evidence of a coordinating action between storage lipid synthesis and phospholipid biosynthesis, which relates to this close relationship and share of main synthetic precursors (Gaspar et al., 2011).

## Regulation of lipid metabolism

The metabolic network for biosynthesis of different lipids is regulated by a variety of mechanisms, such as regulation of gene expression from both cis- and trans- acting elements, (Lopes and Henry, 1991; Schüller et al., 1992) protein phosphorylation (Chumnanpuen et al., 2012; O'Hara et al., 2006; Ratnakumar et al., 2009; Shi et al., 2014) and protein-lipid binding mechanisms (Hofbauer et al., 2014; Loewen et al., 2004). Furthermore, lipid metabolism regulation can be affected by a multitude of external factors such as carbon source and nutrient availability, growth stage, pH and temperature (Carman and Henry, 1999; Gaspar et al., 2007). A comprehensive overview of all the regulation mechanisms with a role in these aspects would fall out of scope of the work developed here, so instead the focus will be directed towards mechanisms that directly impact important metabolic pathways and enzymes in fatty acid biosynthesis and downstream lipid metabolism which had a role in the results later observed. These are composed of the most well understood regulation pathways of lipid metabolism and involve inositol-sensing regulation mechanisms, mechanisms dealing with PA-related signaling and control of acyl-CoA levels.

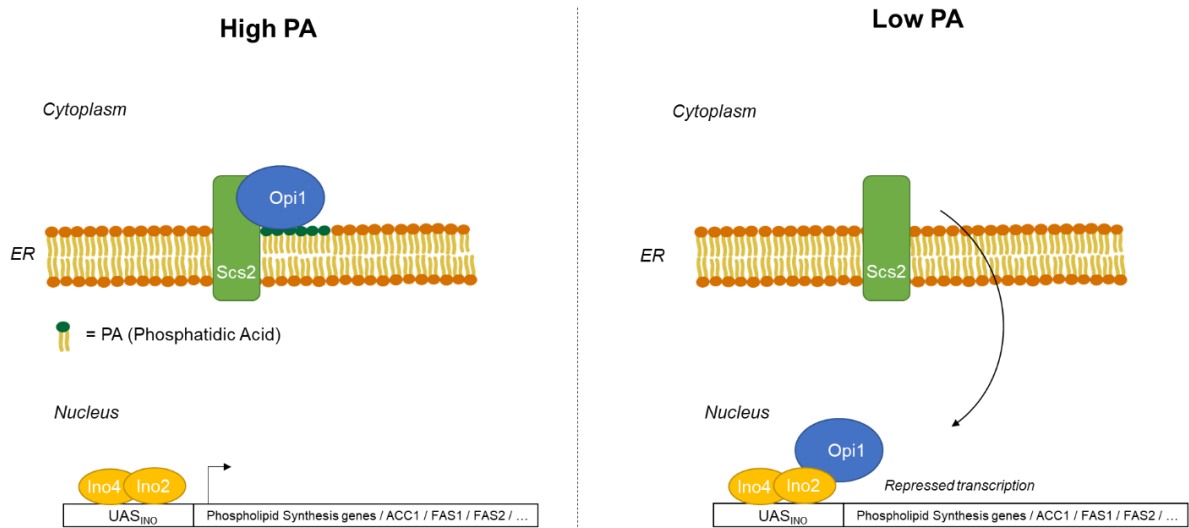
### UAS<sub>INO</sub>-mediated regulation

Probably the most important and well understood mechanism of phospholipid regulation that spans to other pathways of the lipid metabolism network is the transcriptional regulation mediated by the Inositol-responsive Upstream Activating Sequence (UAS<sub>INO</sub>). This sequence is a short repeating element (consensus 5'-CATGTGAAAT-3') that was first found on the promoter of the Inositol-3-phosphate synthase gene *INO1* (Lopes and Henry, 1991). The sequence is a binding site for the Ino2/Ino4 complex, which activates gene expression on genes downstream of this element.

The UAS<sub>INO</sub> element is found in the promoter region of many genes involved in phospholipid biosynthesis belonging to both the CDP-DAG pathway such as *CDS1*, *CHO1*, *PSD1*, *CHO2* and *OPI3* but also the Kennedy pathway like *EK11*, *EPT1*, *CK11* and *CPT1* (Chen et al., 2007;

Wimalaratna *et al.*, 2011). Besides this direct strong correlation with phospholipid biosynthesis, the UAS<sub>INO</sub> element is also found on the genes coding for the fatty acid biosynthesis machinery, *ACC1*, *FAS1* and *FAS2* (Chirala, 1992; Schüller *et al.*, 1992), being therefore an important holistic element of lipid metabolism regulation.

Opi1 is a repressor of the Ino2/Ino4 complex. In this sense, by binding to Ino2 and inhibiting its activity, Opi1 acts as a transcriptional regulator of UAS<sub>INO</sub>-containing promoters, regulating phospholipid and fatty acid synthesis (Chen *et al.*, 2007). The function of Opi1 is controlled by its cellular localization, i.e. it can be found associated with the ER membrane when inactive, and it is translocated to the nucleus in order to bind to Ino2 and repress its transcription factor activity (Loewen *et al.*, 2004). Binding of Opi1 to the ER membrane is mediated through binding to the integral membrane protein Scs2 and this binding is stabilized by the interaction with PA present in the membrane (Loewen *et al.*, 2004, 2003) (**Figure 7**).



**Figure 7. UAS<sub>INO</sub>-mediated regulation.** The transcription factor complex Ino2 and Ino4 bind to the UAS<sub>INO</sub> regulatory element present in the promoter region. In conditions of high PA, Opi1 is mostly bound to the ER membrane through Scs2 where PA is enhancing this interaction. When PA concentrations are low, the interaction strength of Opi1 with the ER membrane is decreased, and this factor is transported to the nucleus where it binds to Ino2, inhibiting expression of phospholipid and fatty acid biosynthesis genes (adapted from Henry *et al.*, 2014).

The levels of PA in the membrane will directly impact the association of Opi1 to the ER, and therefore severely impact the regulation of important factors in lipid metabolism, giving PA a central role in lipid metabolism regulation (Carman and Henry, 2007). In this sense, high levels of PA increase the number and strength of the interactions between Opi1 molecules and the ER, whereas reduced PA levels allow Opi1 to be released from the membrane and to be transported into the nucleus, where it attenuates transcription of UAS<sub>INO</sub>-regulated genes by

binding to Ino2. Furthermore, Opi1 has been shown to more effectively bind PA having C16 over C18 fatty acids in its composition, allowing it to act in chain length control of the produced fatty acids through modulation of Acc1 activity (Hofbauer et al., 2014).

## The role and regulation of Pah1

As seen before, PA levels have a critical role in regulating lipid metabolism. The phosphatidic acid phosphatase Pah1, a homologue of the human lipin gene, dephosphorylates PA into DAGs that can then be used for TAG synthesis or for phospholipid synthesis through the Kennedy pathway (Han et al., 2007). Since it has this ability to convert PA into DAG, Pah1 is thought to be the main regulator of PA levels, therefore able to impact regulation of both general fatty acid and phospholipid biosynthesis pathways (Carman and Han, 2009; Chen et al., 2007). Furthermore, variations in levels of PA have been shown to impact ER membrane structure (Han et al., 2008a) and deletion of the *PAH1* gene has been associated with formation of enlarged ER membrane structures (Santos-Rosa et al., 2005).

Pah1 can be phosphorylated *in vivo* in multiple sites and this phosphorylation has a negative effect on the protein function (O'Hara et al., 2006). Dephosphorylation of Pah1 is carried out by the phosphatase activity of the Nem1-Spo7 complex (Santos-Rosa et al., 2005). The Nem1-Spo7 complex is localized in the ER membrane and Pah1 can be found in the ER membrane associated with this complex, coinciding with sites of LD biogenesis. Studies so far have shown that the interaction of Pah1 with the ER membrane is dependent on an amphipathic helix at the N-terminus and that exposure of this helix depends on the phosphatase activity of the Nem1-Spo7 complex (Karanasios et al., 2010).

The activity of Pah1 has also been shown to be affected by other metabolites. Presence of the phospholipids PI, CL and CDP-DAG have been shown to increase its activity while sphingoid bases and higher levels of the nucleotides ATP and CTP have been shown to inhibit its activity (Carman and Han, 2009).

## Acyl-CoA feedback regulation

As stated before, acyl-CoA is the direct product of the fatty acid biosynthesis machinery and a central substrate for many lipid biosynthetic pathways (Nees et al., 2015). These features make it important for the cell to regulate acyl-CoA levels in order to keep a lipid homeostasis at a cellular level.

Biosynthesis of acyl-CoA is self-regulated. The fatty acid synthase complex in yeast is inhibited by the acyl-CoA it produces (Sumper and Träuble, 1973) and increased levels of long chain acyl-CoA have been related to lower acetyl-CoA carboxylase activity, even though the

exact mechanism for this is not fully described (Faergeman and Knudsen, 1997; Kamiryo et al., 1976; Kamiryo and Numa, 1973; Wakil and Abu-Elheiga, 2009). It is possible however, that an allosteric regulation exists for Acc1 in which this is inhibited by acyl-CoA, since this inhibition was observed for the acetyl-CoA carboxylase from rat liver (Ogiwara et al., 1978). This regulation of Acc1 activity dependent on the levels of acyl-CoA allows for a feedback regulation mechanism working in a fashion that when acyl-CoA is available at increased levels, fatty acid biosynthesis activity is decreased resulting in less acyl-CoA production. This mechanism is responsible for inhibition of the fatty acid biosynthesis machinery when high levels of free fatty acids are detected, as an example, when fatty acids are supplied in the medium (Black and DiRusso, 2007; Kamiryo et al., 1976). Free fatty acids are activated to acyl-CoA by the fatty acyl-CoA synthases Faa1-4 and Fat1 (Black and DiRusso, 2007; Faergeman et al., 2001) and therefore high levels of free fatty acids consequently translates into an increase in acyl-CoA, which in turns acts through feedback inhibition of the fatty acid biosynthesis pathway.

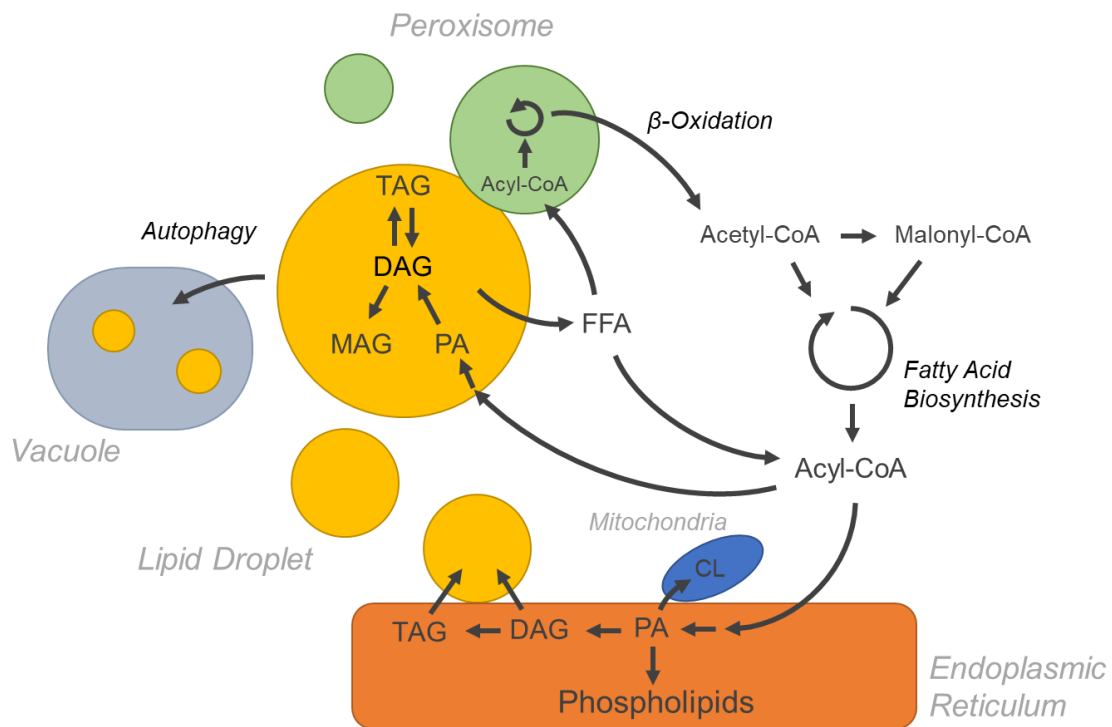
The levels of acyl-CoA have also been shown to be related to the amount of acyl-CoA binding protein Acb1 in the cell in a proportional way, since overexpression of *ACB1* has been correlated to increased acyl-CoA levels (Knudsen et al., 1994). Also, the activity of Acb1 has been shown to be related to the regulation mechanism of different genes involved in fatty acid and phospholipid biosynthesis, since deletion of the *ACB1* gene or replacing with a mutant with no acyl-CoA binding activity increased expression levels of *FAS1*, *FAS2* and *ACCI*. This change in expression levels highlights a direct effect at the transcriptional level mediated through acyl-CoA binding to Acb1 and that the regulation of acyl-CoA levels is therefore mediated by the acyl-CoA-Acb1 link (Feddersen et al., 2007).

## The subcellular organization of lipid metabolism

When looking at the lipid metabolic network, it is often easy to fall into the perception of a linear set of reactions and metabolites simply interconverting to each other. In reality, lipid metabolism occurs through different spaces among different organelles found in the cell (Henry et al., 2012). While fatty acid biosynthesis occurs in the cytosol, acyl-CoA is thought to be transferred either directly to membranes by the FAS complex (Sumper and Träuble, 1973) or to other organelles by the acyl-CoA binding protein Acb1 to participate in different processes (Knudsen et al., 1994). As shown in **Figure 8**, lipid metabolism is partitioned mostly among the Endoplasmic Reticulum (ER) and LDs (Grillitsch et al., 2011; Henry et al., 2012). Mitochondria have a relevant role in cardiolipin synthesis (Gohil et al., 2004) and peroxisomes and vacuoles are involved in degradation of LDs and fatty acid oxidation (Sibirny, 2016; Teter et al., 2001; van Zutphen et al., 2014). At the ER, PA is produced from cytosolic acyl-CoA and glycerol-3-phosphate as described before. PA is then either further converted to other phospholipids in the ER or is used towards TAG synthesis (Czabany et al., 2007). Production of DAGs from PA can either happen in the ER, where these are converted into TAGs here



during the LD assembly process as it will be explained further ahead, or TAGs can be synthesized *de novo* from glycerol and acyl-CoA exclusively in the LD (Athenstaedt and Daum, 1997).



**Figure 8. Major organelles involved in lipid metabolism in yeast.** Lipid metabolism is spread among the cytoplasm, Endoplasmic Reticulum (ER), Lipid Droplet (LD), Peroxisome and Vacuole. Fatty acid biosynthesis is a cytosolic process. Produced acyl-CoA from the FAS complex can be transported to the ER for phospholipid and neutral lipid synthesis or to lipid droplets also for neutral lipid synthesis. Neutral lipid can be hydrolysed either through autophagy and transport to the vacuole or through neutral lipid hydrolysis by lipases with the release of free fatty acids (FFAs), which need to be then reactivated to acyl-CoA for oxidation in the peroxisome.

LDs have a close relationship with the ER (Barbosa et al., 2015a; Schuldiner and Bohnert, 2017). They are assembled from the external ER membrane and remain associated with it during this process. As a result, DAGs and TAGs formed in the ER are transferred to LDs during the LD assembly process and while this organelle is associated with the ER membrane (Jacquier et al., 2011). LDs can fully synthesize PA *de novo* from glycerol and acyl-CoA through the action of Slc1 and Gpt2 (Athenstaedt and Daum, 1997) and acylate DAGs to TAGs through Dga1 and Lro1 (Sandager et al., 2002). Assuming cytosolic Pah1 can interact with the external lipid droplet phospholipid membrane, it is also able to dephosphorylate PA to DAG and therefore LDs have the full machinery for synthesis of TAG from glycerol and acyl-CoA. LDs also contain TAG hydrolysis enzymes, the TAG lipases Tgl3, 4 and 5 that are capable of

hydrolysing TAGs into DAGs and FFAs (Athenstaedt and Daum, 2005; Schmidt et al., 2013). Some of these lipases, like Tgl3, are also able to hydrolyze DAGs into monoacylglycerols (MAGs), which can then be cleaved into glycerol and FFAs by the MAG lipase Yju3 (Heier et al., 2010).

When it comes to oxidation of lipids, the peroxisomes take a central role. Presence of external fatty acid supply as well as low nutrient conditions activate peroxisome proliferation through the transcription factors Adr1 and Oaf1-Pip2, which bind to the oleate responsive element (ORE) (Hiltunen et al., 2003; Rottensteiner et al., 2003; Trzcinska-Danielewicz et al., 2008). Peroxisomes have a main function of hosting the  $\beta$ -oxidation pathway which oxidizes long chain acyl-CoAs into acetyl-CoA units that are then through conversion into C4 organic acids used for energy and biosynthetic purposes (Kunze et al., 2006; Poirier et al., 2006). Peroxisome membranes have transport systems for acyl-CoA as well as mechanisms for simultaneous transport and activation of cytosolic FFAs into peroxisomal acyl-CoAs (Chen et al., 2012; Hiltunen et al., 2003). These organelles can be found associated with LDs and this is thought to be connected with neutral lipid hydrolysis and consequent oxidation of released fatty acids (Binns et al., 2006; Kohlwein et al., 2013; Schuldiner and Bohnert, 2017).

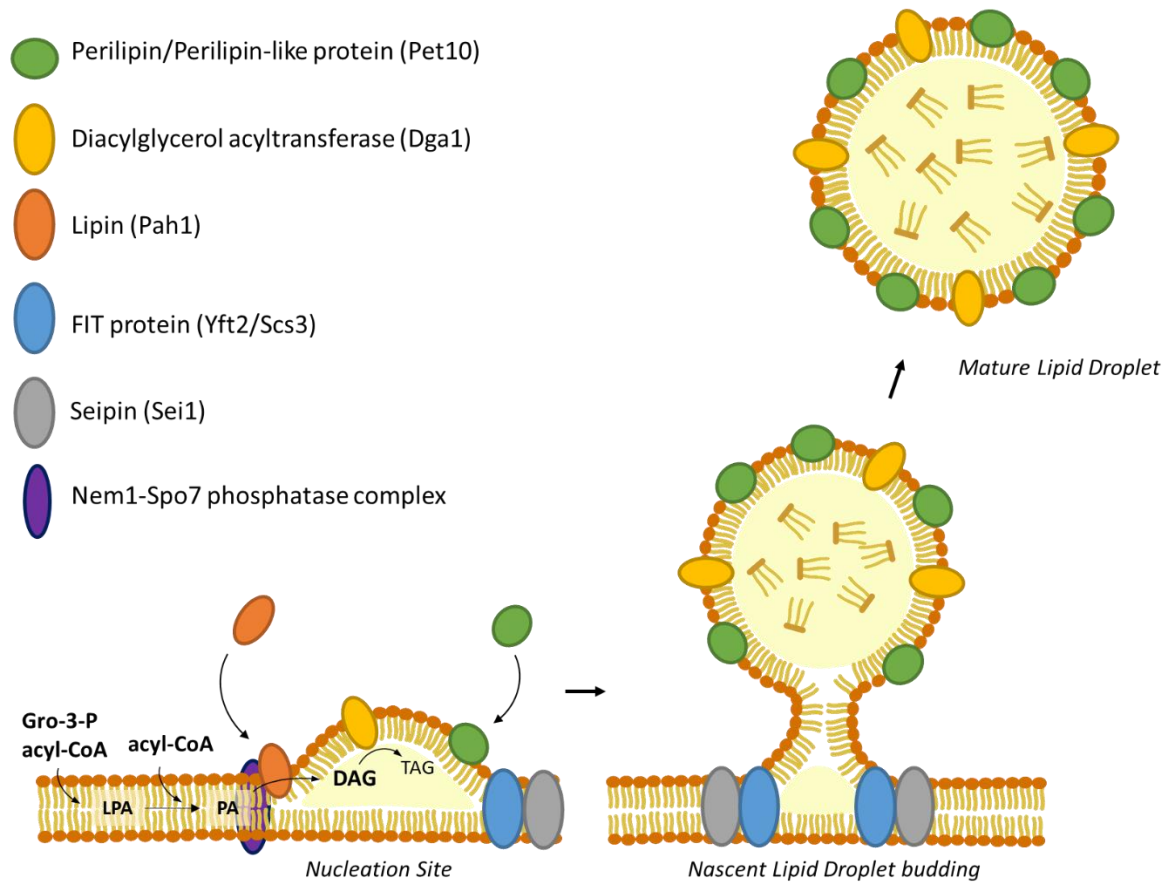
Lipid droplet degradation can also be taken upon through autophagy mechanisms. This requires formation of autophagosome structures with consequent transport to the vacuole, where LDs are the target of hydrolysis (Kaushik and Cuervo, 2015; Singh et al., 2009; van Zutphen et al., 2014). Even though the autophagic process of LD degradation still remains partly unknown, an autophagy-specific lipase, Atg15, has been associated with TAG hydrolysis in the vacuole and is therefore a main player in this process (Maeda et al., 2015).

## The lipid droplet assembly mechanism

Lipid droplets are central organelles in lipid metabolism. Previously thought as a mere structure for storage of neutral lipids, this organelle has recently gotten increased attention due to emerging knowledge of its new functions and associations (Beller et al., 2010; Welte, 2015). The LD is an organelle composed of a neutral lipids core, where the most prevalent lipids are TAGs and SEs, surrounded by a single phospholipid layer, where the polar part of phospholipids faces the cytosol and the hydrophobic part is turned towards the neutral lipid core (Brasaemle and Wolins, 2012). In *S. cerevisiae* it is possible to find associated with this phospholipid membrane more than 50 different enzymes and other proteins (Grillitsch et al., 2011) that turn the LD from a warehouse into a functional distribution center of lipid metabolism (Kohlwein et al., 2013).

LDs are formed in the ER membrane (Joshi et al., 2017) (**Figure 9**). The assembly process starts with generation of a “nucleation site” where a bending in the external ER membrane is associated simultaneously with appearance of a hydrophobic space between the two phospholipid layers (Thiam and Beller, 2017). This site accommodates neutral lipids such as

DAGs and TAGs that are formed in the ER membrane through the action of Pah1 and Dga1 (Wilfling et al., 2013). Pah1 is dephosphorylated by the phosphatase complex Nem1-Spo7 and recruited to the ER membrane in an interaction that is associated with (and necessary to) the emergence of LD assembly sites (Adeyo et al., 2011; Barbosa et al., 2015b). Dga1 on the other hand is known to be located in the ER membrane when LDs are absent and relocate to LDs when these are formed. This transport mechanism is independent of vesicle transport mechanisms and it is suggested to happen by diffusion through the ER membrane to the LD during LD biogenesis (Jacquier et al., 2011).



**Figure 9. Factors involved in lipid droplet biogenesis from the ER.** Nascent lipid droplet assembly requires production of neutral lipid in the ER, during which Pah1 is recruited to the ER membrane by the phosphatase complex Nem1-Spo7. FIT proteins and seipins are crucial structural elements in LD assembly while perilipins and DGATs are also recruited to the nascent LD site during the process. Seipin is present in LD-ER contact sites and allows for detachment of the LD from the ER. LDs are also matured through incorporation of more fatty acids as TAGs through their life cycle. In the mature droplet, perilipins can be found coating it and can be important for maturation, stabilization and mobilization of these.

Besides the metabolic pathways that create neutral lipids fundamental for the formation of LDs, there are other structural factors that allow this process to occur (Chen and Goodman, 2017). The 3 main protein families known to have an important role in structural aspects of LD biogenesis are perilipins, Fat-Inducing Transmembrane (FIT) proteins, and seipins (Chen and Goodman, 2017).

Perilipins are a family of proteins characterized by the presence of a conserved PAT (Perilipin/ $\Delta$ DRP/TIP47) domain (Bickel et al., 2009). The action of perilipins has been well described in mammals. In humans, 5 different perilipin proteins exist, and these have different functions (Sztalryd and Brasaemle, 2017; Sztalryd and Kimmel, 2014). Perilipin proteins can be involved in controlling lipase action as it is the case for Perilipin 1, which depending on its phosphorylation state can either promote or inhibit the binding of lipases to the LD (Brasaemle et al., 2000; Marcinkiewicz et al., 2006). Other perilipins, such as Perilipin 3, are involved mostly with the biogenesis process of the LD, having a role in the assembly process of the LD rather than in the protection against hydrolysis after maturation (Bulankina et al., 2009).

FIT proteins have been described as transmembrane ER proteins that localize to LD biogenesis sites and actively participate in this process (Choudhary et al., 2015). Deletion of the FIT2 homologue genes in *S. cerevisiae*, *YFT2* and *SCS3*, leads to accumulation of LDs inside the ER lumen, suggesting a lack of a budding ability by the ER membrane to form the LD (Choudhary et al., 2015). It is known that knockout of FIT genes impairs LD formation (Kadereit et al., 2008; Miranda et al., 2014) and overexpression usually leads to improved accumulation and formation of LDs (Cai et al., 2017; Tan et al., 2014). In terms of structure and function, it is only known that both FIT1 and FIT2 proteins have six transmembrane domains with both N- and C-termini localized to the cytosol (Gross et al., 2010) and FIT protein's ability to bind TAGs is important for their LD formation role (Gross et al., 2011). Apart from these factors, nothing else is known about the mechanism of action of these proteins and their role in LD assembly.

Seipin is an integral ER membrane protein with two transmembrane domains. Deletion mutants of the yeast seipin gene *SEI1* reveal abnormal phenotypes of LD structures where some cells contain one or a few supersized LDs while others showed an amorphous aggregation of several small LDs (Fei et al., 2008; Szymanski et al., 2007). It is accepted that seipins have an important role in LD morphology (Cartwright et al., 2015) and have a major role in stabilization of LD-ER contact sites (Grippa et al., 2015). The amorphous LD morphology associated with *SEI1* deletion has been shown to be related with envelopment of LDs by the ER membrane due to de-regulation of promoting correct contact sites (Grippa et al., 2015).

Even though LD assembly has been a recent focus of research, not much is known so far on the exact mechanism through which this occurs and many questions still remain unanswered as to the role of the different elements such as FIT proteins or seipins.

# Objectives and Overview

This thesis aims to better understand aspects of *S. cerevisiae* lipid metabolism in the context of its developing potential as a cell factory for production of oleochemical precursors. Through analysis of the diagram in **Figure 1** representing basic oleochemical interconversions, the most interesting precursors to produce in terms of their versatility for application in industry are TAGs and free fatty acids. This is due to their potential to be chemically converted to virtually any other basic oleochemical species. Furthermore, the advancements in engineering *S. cerevisiae* for production of basic oleochemicals has had more success with TAGs and free fatty acids than with any of the other chemicals.

As such, this thesis is divided into two main parts. In the first one, composed of **papers I to III**, aspects of fatty acid metabolism were studied in order to better understand the dynamics and pathways underlying free fatty acid production. For this, previously described genetic alterations that result in improved free fatty acid production were applied, which allowed for studying the effects arising from these modifications or combinations of them. In the second part, *S. cerevisiae* was engineered for the highest TAG production yield reported so far, which is divided between **paper IV and V**. The two papers represent different strategies for achieving this. In **paper IV**, TAG production was improved through increasing the flux through fatty acid biosynthesis and the TAG synthesis pathway, as well as deleting competing pathways. **Paper V** describes a novel strategy for improving TAG production levels. This consisted of enhancing the LD biogenesis and assembly process in order to facilitate TAG accumulation on this organelle.

The work developed here represents a process of simultaneous engineering of cell factories coupled with a scientific approach towards better understanding of how the system responds to the changes applied.



# Methodology for Analysis of Lipid-producing Cell Factories

## Detection, screening and analysis

Designing and building microbial cell factories heavily relies on the ability to analyze the result of the engineering process. As such, an ability to detect presence of a product and correctly quantify it is vital for the development of metabolic engineering as the field relies on reproducibility of results and ability to compare absolute values between different studies (Endy, 2005). Furthermore, accurate measurements will impact any calculations of yield, productivity, and even cost-effectiveness of the process, being again fundamental in the application aspect of the technology.

The choice of method to analyze a given construct will depend largely on the objectives of the study and the stage of development of such strain and product (Hounslow et al., 2017).

For proof of concept studies where a given molecule is being produced for the first time, it is important to be able to detect it with high sensitivity, since this will probably be firstly produced only in trace amounts. At this stage, large method development times are to be expected for optimization of instruments and protocols in order to specifically distinguish the compound of interest.

When more knowledge is available about a certain metabolite, pathway or physiology surrounding the compound of interest, there are two main types of assays that can be performed depending on the study objective: either screening or analytics. Screening is related to high throughput methods for strain construction, such as mutation libraries for a gene, analysis of randomly mutated strains or expression of cDNA libraries (Dietrich et al., 2010). Here one needs to identify single clones from thousands or millions of others, and therefore requires a range of sensitivity that allows to discriminate clones. Absolute quantification is not a priority in a screening approach, but simply fast analysis of all clones to separate the ones that have a higher or lower relative signal. As for analytics, these are usually time consuming, single-sample procedures that commonly allow for thorough analysis of the different metabolites in one single strain, with accurate absolute or relative quantification (Khoomrung et al., 2013).

The descriptions here do not intend to be a thorough review of available methods but more as a guideline introduction to understand the purpose and differences of the methods applied during the work developed here. Naturally, a higher focus is put into the lipid analytics methods since these were the ones used here.

## Screening methods

As a simple description, screening in design of cell factories with the objective to improve production can be done through detection of the product or detection of a certain metabolite precursor. For high throughput applications, the detection of the compound in question must be done in a simple and fast fashion, for which colorimetric and fluorimetric assays are usually a good solution since these can be easily detected and registered by optic sensors (An, 2009).

A common approach to screening for lipid content in a cell is staining with a fluorescent dye. Dyes like BODIPY or Nile Red bind and emit fluorescent light upon binding to neutral lipids, but do not emit fluorescence in solution (Govender et al., 2012). This makes them ideal for visualizing internal lipid structures through fluorescent or confocal microscopy. Staining of a culture and sorting different clones based on emitted fluorescence values can also be done and has been applied before in a medium-throughput fashion (Govender et al., 2012; Shi et al., 2016). The limitation in this system comes when factors such as staining efficiency and dye diffusion come into play, creating large noise variability between cells and possibly occluding the possibility of single-cell sorting efficiency, which hinders single-cell applications for high-throughput screening.

Another more sophisticated and sensitive way to detect metabolites is through the use of biosensors. Briefly explained, biosensors are *in vivo* molecular systems that can sense a certain compound/metabolite and relate this to an output signal, usually expression of a fluorescent protein, with a dynamic range in which expression levels are related to metabolite concentration (Yan and Fong, 2016). One advantage of biosensors is that these can very efficiently detect intracellular metabolites *in vivo* and report with precision transient or unstable compounds that are hard to work with *in vitro*. But the main advantage is the ability to detect single-cell signals using techniques such as flow cytometry and select individual cells as well-performing clones among thousands or millions of them in a matter of minutes (Piatkevich and Verkhusha, 2011). Limitations of biosensors come with the need for a specific recognition of the metabolite to be sensed, for which a molecular system needs to already exist or be possible to create from existing systems where the target molecule is recognized.

With this in consideration, biosensors have been successful when it comes to detection of -CoA molecules. Biosensors able to detect intracellular variations of acyl-CoA (Teo et al., 2013) or malonyl-CoA (David et al., 2016; Ellis and Wolfgang, 2012) levels have been successfully designed in *S. cerevisiae* and are a powerful tool for the development of oleochemical producing cell factories.



## Methods for lipid analysis

Lipidomics is a science with many different methods available that allow for analysis of different parameters when it comes to the lipids in a given sample. Different methods, instruments and sample preparation protocols will provide different information and what to use depends on the objective of the study.

### Sample preparation dictates target analysis

Sample preparation is the process in which the metabolites to be analyzed are separated from the rest of the sample impurities and are prepared to be detected by the instrument in question. In case of lipid analysis, extraction of lipids is usually done using organic solvents, which provide a hydrophobic phase towards which lipids can diffuse and separate from all the soluble parts of the cell (Lee et al., 2010). Cell disruption and contact with organic solvents will extract lipids from all membranes, unless organelles are separated previously in some way, as for example, ultracentrifugation.

The conditions in which this extraction is done will dictate if different lipid classes remain intact or if fatty acids will be hydrolyzed or parts of phospholipids oxidized. In a case where the total fatty acid composition or quantification of all fatty acids in the cell is desired, all lipid classes are hydrolyzed and fatty acids are usually trans-esterified to fatty acyl methyl esters (FAMES) (Khoomrung et al., 2012; Rodríguez-Ruiz et al., 1998). In our studies, we have sometimes used transesterification of total fatty acids into FAMES and analyzed these in a GC-MS (Gas Chromatography coupled to detection through Mass Spectrometry) or GC-FID (Gas Chromatography coupled to detection through Flame Ionization Detector) instrument in order to analyze total fatty acid production in the cell and the composition of these fatty acids. Separation through a GC wax column allows for separation of fatty acids with different chain lengths and desaturation levels. By running in the same batch a set of external standards, it is possible to quantify the concentration of each fatty acid species through correlation with the standard curve of the “MS ion current” versus “known concentration of fatty acid”. This can give valuable information on quantifying i) the fatty acid biosynthesis pathway activity, since all fatty acids in the cell, independent of lipid classes, are produced via this pathway, ii) variations in fatty acid saturation levels and iii) fatty acid chain length, whether this is from the FAS system or from the elongation system.

In a case where lipid classes need to remain intact, the extraction conditions should be milder and controlled (Hounslow et al., 2017). Lipid classes are extracted also by the use of organic solvents and analyzed directly. In our studies, we often use this approach in combination with an HPLC-CAD (High Performance Liquid Chromatography coupled to detection through Charged Aerosol Detection) analysis, where lipid classes are separated through a reverse-phase liquid chromatography column according to their polarity and are detected by a current detector that is sensitive to the concentration of that given lipid class (Khoomrung et al., 2013). Again

by running in the same batch a set of external standards of known concentrations, it is possible to quantify each lipid class through correlation with the standard curve of the “CAD current” versus “known concentration of lipid”. The HPLC-CAD method here applied does not allow, however, to know the fatty acid composition of the lipids in each lipid class. More sensitive methods exist based on LC-MS/MS detection, that allow to separate between chain length composition and saturation level of each different lipid species and infer fatty acid composition (Mehlem et al., 2016). Another more labor-intensive alternative for analysis of lipid class composition is separation by LC as done in our HPLC-CAD method or by SPE (solid phase extraction), but each peak should be collected and esterified to FAME for GC-MS analysis (Avalli and Contarini, 2005). One of the issues of milder extraction conditions for lipid class analysis is the possibility of incomplete cell disruption and extraction, which results in underestimated values for each lipid class.

Protocols for extraction and isolation of specific species are also available. In cases where we wanted to quantify free fatty acids without pulling fatty acids from other lipid species, such as in **paper II and III**, a specific solvent mix was used that was shown to specifically esterify this fatty acid pool with methanol, forming FAMEs that could then be eluted in hexane separately from other lipids (Haushalter et al., 2014).

## Instrumental setup

The choice of analytical instrument and the components of it is dictated by the metabolites that are being targeted. GC is able to separate volatile metabolites based on their boiling points and their chemical interaction with the stationary phase of the column. GC has some advantages over LC such as the resolution level one can acquire with the technique. Peak separation in GC is usually more accurate and reliable than LC. Other factors to consider is the time of each run, in which GC runs are usually faster than LC, and the cost of each run, given that solvent costs for LC are also often much higher than the costs of the carrier gas in GC. However, the use of GC requires the metabolites to be in a gas phase, so these must be volatile or converted into a volatile form before being run. In case of fatty acids for example, these need to be derivatized to methyl esters (FAMEs) previous to a run in GC because these cannot enter a gas phase easily.

The choice of the gas chromatography method is also dependent on the metabolites to be analyzed. Proper programming of the GC oven temperature over the analysis allows for separation of different analytes with a wide range of boiling points such as fatty acids with different chain lengths. A too low temperature would for analytes with high boiling points lead to very high retention times and elution without a proper signal-to-noise ratio. A high column temperature would separate peaks of high boiling point analytes, but lower analytes would be covered by the solvent tail. Through **paper I**, the analysis needed to be focused on short chain fatty acids rather than long chain, which required a setup of the temperature variation specific to separate the shorter chains efficiently.

In terms of analyte detection, we have through this work used either a MS or a FID post GC separation. Detection through MS allows for selective and specific detection of FAMES and identification of each different species by the mass spectra ion profile of each FAME species. This way, the detection sensitivity is highly increased since one can scan for specific ion detection, defined as selective ion monitoring (SIM). As an example, here we have used for many samples detection of a broad range of ions with an  $m/z$  range of 50-650  $m/z$  and simultaneously run analysis in SIM mode with the ions at  $m/z$  55, 67, 74, and 79 since these were previously identified as specific ions for FAMES of any chain length. This way, high sensitivity and accuracy in fatty acid detection was possible in cases where this was needed.

Concepts described in this last section regarding GC-MS setup for proper metabolite separation from extracted samples are extensively revised in (McMaster, 2008).



# Part I: Understanding Free Fatty Acid Metabolic Fluxes

## Free fatty acids - a versatile precursor

Referring back to the diagram depicting interconversion between different basic oleochemicals (**Figure 1**), the value of FFAs is evident when it comes to their application versatility for conversion into other basic oleochemical species. FFAs can be chemically converted into fatty acid methyl esters, fatty amines or fatty alcohols (Salimon et al., 2012), being therefore a possible first precursor for virtually any oleochemical product.

Fatty acids are industrially produced through hydrolysis of TAGs, but direct microbial production of these molecules without chemical hydrolysis of TAGs offers many advantages. Besides the aforementioned sustainability improvements from shifting from exotic plant extraction of precursors towards microbial fermentation, metabolic engineering offers the possibility of producing tailored fatty acids with desired chain lengths and saturation levels. In terms of downstream processing, microbial production of FFAs does not produce glycerol as a side product and FFAs can be secreted to the culture medium, which due to their hydrophobic properties simplifies extraction through a two phase liquid separation process.

## Metabolic engineering for free fatty acids and fatty acid-derived products

Microbial cells can be engineered for production of either FFAs or other fatty acid-derived oleochemicals (Pfleger et al., 2015). *S. cerevisiae* has been engineered for production of basic oleochemicals like FFAs, fatty alcohols and fatty acid ethyl esters as well as hydrocarbons such as alkanes and alkenes. In the following section some of the more relevant strategies will be discussed.

## Free fatty acid production pathways and strategies

FFAs in *S. cerevisiae* have been suggested to be mainly a product of lipid remodeling (Scharnewski et al., 2008). Since *S. cerevisiae* is not known to have cytosolic thioesterases that

would act in the hydrolysis of acyl-CoA esters to release FFAs, cytosolic FFAs can theoretically be a product of either i) neutral lipid hydrolysis through sterol esterases such as Yeh1, Yeh2 or Tgl1 (Köffel et al., 2005) or TAG lipases such as Tgl3, 4 and 5 (Athenstaedt and Daum, 2005) or ii) phospholipid hydrolysis through the action of phospholipases B such as Plb1, 2, 3 or Nte1 (Merkel et al., 1999).

Formed cytosolic FFAs need to be reactivated to acyl-CoA so that fatty acids can be transported to other processes and organelles. This process is done through the action of fatty acyl-CoA synthases Faa1, 2, 3, 4 and Fat1 (Black and DiRusso, 2007). Faa1 and Faa4 are homologues and the main long-chain fatty acyl-CoA synthetases in the cell (Faergeman et al., 2001). Faa2 is a peroxisomal medium-chain acyl-CoA synthase (Hiltunen et al., 2003; Knoll et al., 1994) while Faa3 has a much lower activity compared to Faa1 and Faa4 (Knoll et al., 1994). Fat1 is a bifunctional enzyme required both for fatty acid transport of long chain fatty acids but also activation of very long chain fatty acids (Zou et al., 2002).

While deletion of either *FAA1* or *FAA4* causes a significant loss of acyltransferase activity, the deletion of one of the genes is to some degree compensated by the other. Simultaneous deletion of the *FAA1* and *FAA4* genes causes a strong phenotype where FFAs are overproduced and secreted to the extracellular medium (Leber et al., 2015; Scharnewski et al., 2008; Zhou et al., 2016b). This phenomenon is believed to be due to an overaccumulation of these species and consequential diffusion to the extracellular media enhanced by an inability to reactivate and re-import released FFAs rather than an active export mechanism (Faergeman et al., 2001).

The deletion of these fatty acyl-CoA synthases is the starting point for most FFA overproduction strategies. Production of FFAs can be increased by about 40% solely by cytosolic expression of the truncated version of acyl-CoA thioesterase gene *ACOT5* encoding the *Mus musculus* peroxisomal acyl-CoA thioesterase 5 (Chen et al., 2014b). This strategy was also the basis of another study (Runguphan and Keasling, 2014) where a truncated *Escherichia coli* thioesterase gene '*tesA*' was expressed in a *Δfaa1 Δfaa4* strain and endogenous genes from the fatty acid biosynthesis pathway *ACC1*, *FAS1* and *FAS2* were also overexpressed under a strong constitutive promoter (*TEF1p*). This strain produced 400 mg/L of FFAs in a mixed carbon source medium (1.8% Galactose, 0.2% Glucose).

Engineering of *S. cerevisiae* by (Zhou et al., 2016b) where *FAA1*, *FAA4* and the gene coding for the first enzyme in the  $\beta$ -oxidation *POX1* were deleted resulted in 580 mg/L of FFAs using 2% glucose in minimal synthetic media (8% of the maximum theoretical yield for FFA production from glucose, assuming this value to be 0.32 gFFA/gGlucose (Caspeta and Nielsen, 2013). Further heavy engineering of this strain with expression of the truncated *E. coli* thioesterase '*TesA*', the fatty acid synthase gene *RtFAS* from the oleaginous yeast *Rhodospiridium toruloides*, the endogenous *ACC1* gene and a partly heterologous citrate-lyase cycle for generation of cytosolic acetyl-CoA consisting of the *M. musculus* acetate-citrate lyase gene *MmACL*, malic enzyme gene *RtME* from *R. toruloides*, endogenous malate dehydrogenase *MDH3* with removed peroxisomal signal and citrate transporter gene *CTP1*, resulted in FFA production levels up to 10.4 g/L in fed batch or 1.1 g/L in shake flask culture

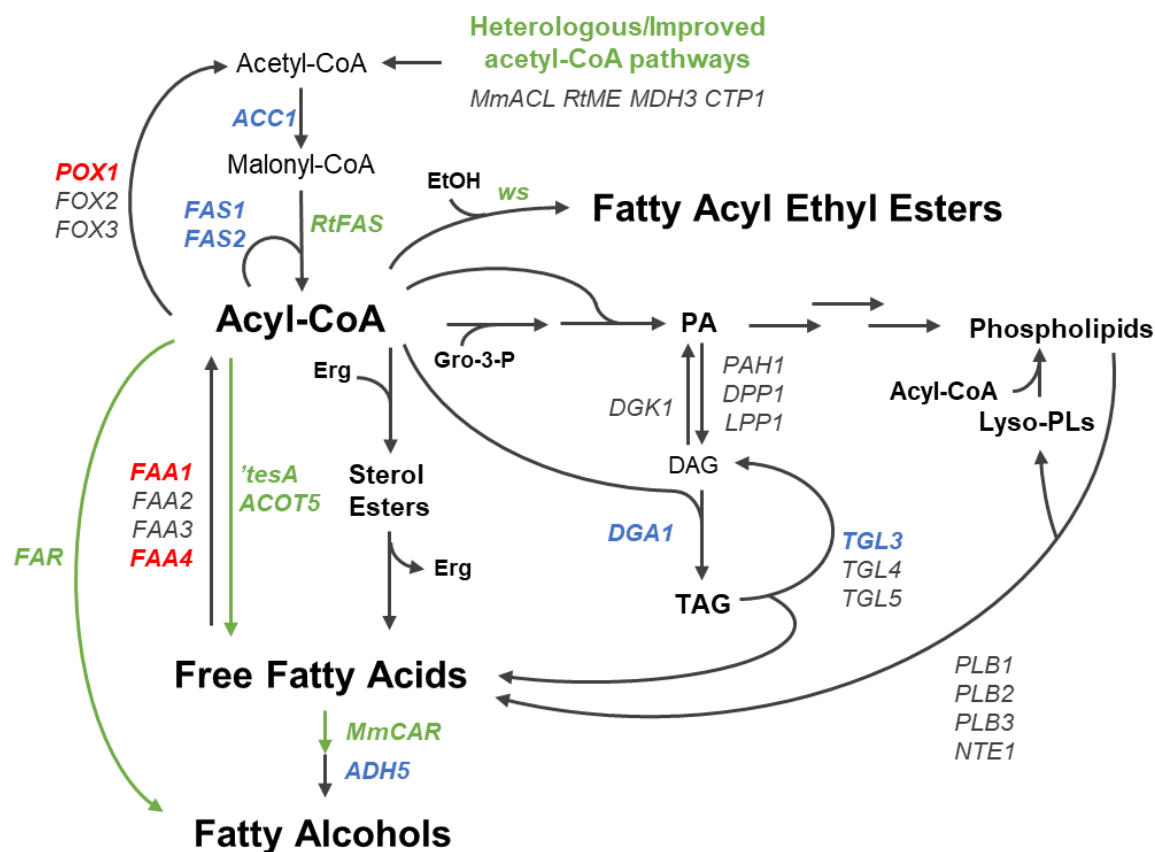
reaching up to 14% of the maximum theoretical yield of FFA production from glucose (Zhou et al., 2016b).

Another strategy by (Leber et al., 2015) generated a background strain with deletion of the acyl-CoA synthase genes *FAA1*, *FAA4*, *FAT1* and *FAA2*, the  $\beta$ -oxidation gene *POX1* and the peroxisomal acyl-CoA transporter gene *PXA1*. In this strain, overexpression of the diacylglycerol gene *DGAI* and the TAG lipase gene *TGL3* allowed for production of FFAs up to 2.2 g/L in complex YPD medium. This strategy used the fluxes towards TAG synthesis and TAG hydrolysis to produce FFAs. Interestingly, overexpression of only *DGAI* or *TGL3* did not increase the FFA titers in this background strain, indicating presence of an overlaying mechanism that regulates the flux to and from this lipid pool, making FFA production through TAG synthesis and hydrolysis only possible if both pathways are overexpressed.

*S. cerevisiae* has also been engineered not only for increased titers of FFA production, but also for production of different fatty acid acyl-chain distribution. Engineering the native *S. cerevisiae* FAS complex by inserting a short-chain thioesterase domain in its structure and mutating the ketoacyl synthase domain to change its substrate specificity resulted in production of short and medium chain (C6-C12) fatty acids up to 175 mg/L, a more than 50-fold increase over wild-type *S. cerevisiae* levels, in which these can only be detected in low concentrations (Zhu et al., 2017).

In another study, overexpression of human FAS mutants together with two different heterologous short chain thioesterases from *Cuphea palustris* (CpFatB1) and *Rattus norvegicus* (TEII) increased production of short chain (C6 to C12) fatty acids to 68 mg/L and additional expression of a phosphopantetheine transferase Sfp from *Bacillus subtilis* resulted in total SCFA titers of up to 111 mg/L (Leber and Da Silva, 2014). A minor improvement was also achieved from another study where the  $\beta$ -oxidation gene *POX1* was replaced by the *Yarrowia lipolytica* gene *POX2*, coding for an acyl-CoA oxidase with a preference for long chain acyl-CoAs. This allowed the strain to oxidize long chain acyl-CoAs into medium-chain acyl-CoAs but not further, transforming long chain fatty acids into C12 fatty acids and increasing short/medium chain fatty acid levels by 3.34-fold compared to a wild-type strain (Chen et al., 2014a).

The spectra of fatty acids produced in *S. cerevisiae* was also expanded towards the very long chain direction. Rewiring of the native fatty acid elongation system through deletion of the *ELO3* gene and overexpression of *ELO1* and *ELO2* and additional expression of a heterologous *Mycobacteria* FAS I system allowed for specific production of C22 fatty acids, which were then used for docosanol (C22 fatty alcohol) production (Yu et al., 2017). Production of very long chain fatty acids through *ELO3* deletion and *ELO2* overexpression was also applied successfully for *in vivo* production of jojoba-like wax esters *S. cerevisiae*, since these are mostly composed of C20 and C22 fatty acids and fatty alcohols (Wenning et al., 2017).



**Figure 10. Overview of native pathways and engineering targets for production of free fatty acids and basic oleochemicals.** Increased production of acyl-CoA was previously enhanced through overexpression of chimeric pathways for generation of cytosolic acetyl-CoA, as well as overexpression of *ACC1*, *FAS1*, *FAS2* and the *FAS* gene from *Rhodospiridium toruloides*. Acyl-CoA can be directly converted to free fatty acids through expression of thioesterases such as the *E. coli* 'tesA or the mouse *ACOT5*. Free fatty acids were also previously overproduced through TAG by overexpressing *DGA1* and *TGL3*. Fatty alcohols can be produced from acyl-CoA through expression of different fatty acyl-CoA reductase (*FAR*) genes, or from free fatty acids through the expression of a carboxylic reductase (*CAR*) and the native alcohols dehydrogenase *ADH5*. Fatty acyl ethyl esters can also be produced from acyl-CoA and ethanol through expression of wax esterases (*ws*). Strategies for production of free fatty acids also rely on deletion of the genes *FAA1*, *FAA4* and *POX1*. Overexpressed native genes are highlighted in blue, heterologous genes in green and deleted genes in red. Reaction arrows in green depict reaction obtained through expression of heterologous enzymes and are not natively existent in *S. cerevisiae*.

## In vivo production of basic oleochemicals



Increasing the capacity of *S. cerevisiae* for overproducing fatty acids might be useful for production of FFAs per se as we have seen in the section before, but it can also be used as a platform strategy for *in vivo* production of fatty acid-derived products. Some basic oleochemicals like fatty alcohols and fatty acid esters can be synthesized *in vivo* from fatty acids through expression of heterologous pathways.

Fatty alcohols are a basic oleochemical, and besides having direct applications, they can be used as an industrial precursor with many applications as it was discussed before. Fatty alcohols can be produced in *S. cerevisiae* by two main pathways. The first pathway uses fatty acyl-CoA as a precursor and the conversion is taken through the action of a fatty acyl-CoA reductase multifunctional enzyme (FAR). The FAR converts acyl-CoA into a fatty aldehyde intermediary which does not leave the enzyme pocket, oxidizing one NADPH to NADP<sup>+</sup> and releasing CoA, and in a second step the fatty aldehyde is reduced to fatty alcohol at the expense of oxidizing another NADPH. The most commonly expressed genes for this reaction have been the *mFAR1* from *M. musculus* (d’Espaux et al., 2017), *TaFAR1* from *Tyto alba* (Feng et al., 2015), and the FAR gene *Maqu\_2220* from *Marinobacter aquaeolei* VT8 (Pfleger and Tyler Youngquist, 2017; Willis et al., 2011; Zhou et al., 2016a). The second pathway for *in vivo* fatty alcohol production uses a carboxylic reductase enzyme (CAR) to convert FFAs to fatty aldehydes at the cost of 1 NADPH and 1 ATP (Akhtar et al., 2013). This pathway requires a second enzyme to reduce the aldehyde group to a primary alcohol (Zhou et al., 2016b). As already mentioned, fatty alcohol conversion has already been coupled to specialty fatty acid synthesis towards production of docosanol and jojoba-like wax esters in which FARs with specificity towards very long chain acyl-CoA were used (Wenning et al., 2017; Yu et al., 2017).

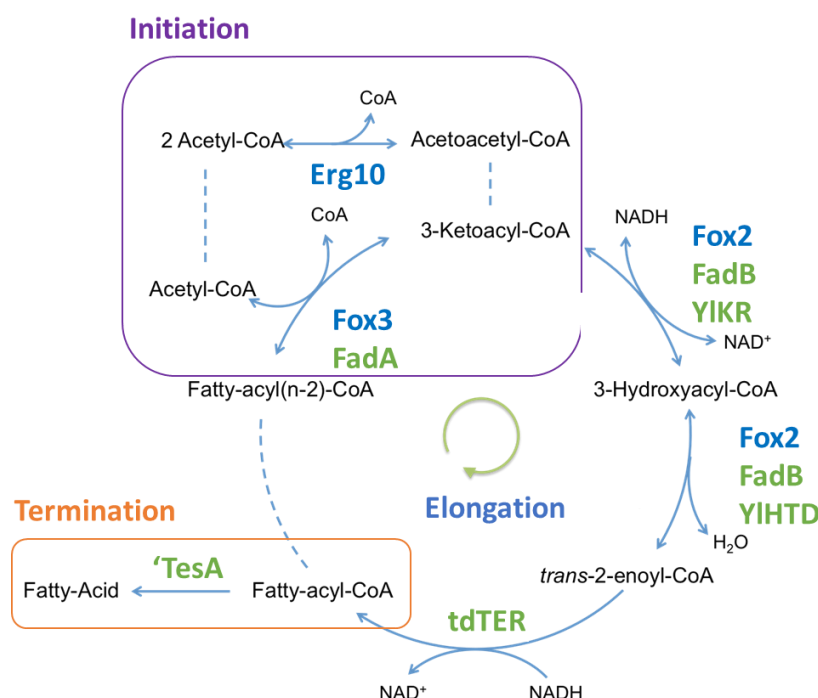
Fatty acid ethyl esters (FAEEs) are chemically similar to fatty acid methyl esters, with the difference that ethanol is used for esterification with the fatty acids instead of methanol. Therefore, FAEEs can be used as basic oleochemicals and directly as a biodiesel offering advantages over FAMES (Yusoff et al., 2014). Production of ethyl esters in *S. cerevisiae* is more promising compared to methyl esters due to its ability to naturally produce ethanol, while methanol is toxic for this cell (Yasokawa et al., 2010). Production of FAEEs in *S. cerevisiae* is enabled through expression of wax ester synthases that can esterify the naturally produced ethanol with acyl-CoA. This has been demonstrated before by combining expression of the *ws2* gene from *M. hydrocarbonoclasticus* DSM 8798 with removal of storage lipids and  $\beta$ -oxidation (Valle-Rodríguez et al., 2014) and through expression of the wax-ester synthase *AtfA* from *A. calcoaceticus* ADP1 (Runguphan and Keasling, 2014). However, the success of this is limited, the highest production titers being 17 mg/L of FAEEs, probably due to a low activity of wax ester synthase enzymes.

## The reverse $\beta$ -oxidation as an alternative to fatty acid biosynthesis

As previously described, the fatty acid machinery composed by the acyl-CoA carboxylase Acc1 and the fatty acid synthase complex encoded by *FAS1* and *FAS2* is highly regulated and limited in its properties. Acc1 is regulated at the transcriptional level by the UAS<sub>INO</sub> element and consequently the Ino2/Ino4/Opi1 system (Chirala, 1992; Hasslacher et al., 1993), at the post-translational level through Snf1 phosphorylation (Shirra et al., 2001; Shi et al., 2014; Woods et al., 1994) and feedback regulated by levels of acyl-CoA (Faergeman and Knudsen, 1997; Wakil and Abu-Elheiga, 2009). Fas1 and Fas2 have been shown to be transcriptionally regulated also by the UAS<sub>INO</sub> element (Chirala, 1992) and have phosphorylation and acetylation sites that might modulate their activity (<http://www.uniprot.org/>). Furthermore, the native *S. cerevisiae* FAS complex synthesizes fatty acids typically with chain lengths from C14 to C18, which is a limitation for production of specialty fatty acids where other chain lengths might be desired. As such, an important advancement point in engineering yeast cell factories for production of oleochemical precursors would be an available alternative pathway for fatty acid synthesis that could offer tailored product modulation and isolated from internal regulatory elements that control fatty acid synthesis rate.

An alternative pathway consisting on the reversal of the fatty acid  $\beta$ -oxidation pathway towards fatty acid biosynthesis was previously designed and successfully applied in *E. coli* (Clomburg et al., 2012; Dellomonaco et al., 2011).  $\beta$ -Oxidation is a fatty acid oxidation pathway where a  $n$ -carbon long acyl-CoA is oxidized to  $n/2$  acetyl-CoA molecules, generating NADH in the process (**Figure 11**). This pathway was successfully engineered in *E. coli* to work in the reverse direction, synthesizing acyl-CoA from acetyl-CoA and NADH.

The pathway design consists of deletion of the first oxidation step in the  $\beta$ -oxidation pathway, where  $O_2$  is used as the redox co-factor for oxidation of acyl-CoA in the first step of the pathway and replacing it with a reversible reaction catalyzed by a trans-enoyl reductase (TER) that uses NADH as co-factor for the reductive reaction. As the other reactions in the  $\beta$ -oxidation pathway are reversible, success of converting the pathway from a fatty acyl-CoA oxidation pathway to a fatty acyl-CoA synthesis pathway in bacteria relies on the shifting of the thermodynamics of the pathway and replacing the only irreversible reaction. In **paper I**, we analyze the thermodynamics of the reverse  $\beta$ -oxidation pathway (**Table 2**) and conclude that it is the replacing of this step by the TER-catalyzed reaction that makes the pathway feasible in the acyl-CoA synthesis direction.



**Figure 11. Schematic representation of the reactions involved on the reverse  $\beta$ -oxidation pathway.** The initiation strategy for formation of acetoacetyl-CoA uses Erg10 (acetyl-CoA acetyltransferase) for the condensation of 2 acetyl-CoAs. Following that, the pathway is composed of Fox3/FadA (3-ketoacyl-CoA thiolase), Fox2/fadB (3-hydroxyacyl-CoA dehydrogenase/enoyl-CoA hydratase multifunctional enzyme) or a combination of YIKR (ketoacyl-CoA reductase) and YIHTD (hydroxyacyl-CoA dehydratase) and tdTER (trans-2-enoyl reductase) for elongation. Termination is performed by cleavage of the fatty acyl-CoA into fatty acids by the thioesterase 'TesA.

Application of this pathway in eukaryotes requires additional concerns regarding compartmentalization. Since  $\beta$ -oxidation in yeast is present in the peroxisome, the pathway needs to be redesigned for cytosolic expression. One single study has successfully applied the reverse  $\beta$ -oxidation in *S. cerevisiae* for butyrate and short chain fatty acid synthesis (Lian and Zhao, 2015). In this study, the *S. cerevisiae*  $\beta$ -oxidation multifunctional enzyme Fox2 did not show *in vitro* activity from cell extracts when expressed in the cytoplasm, indicating that the cytosolic form of this enzyme was not functional. As such, the reversed  $\beta$ -oxidation was built using a chimeric pathway consisting of the *S. cerevisiae* 3-ketoacyl-CoA thiolase Fox3, the *Y. lipolytica*  $\beta$ -ketoacyl-CoA reductase YIKR and  $\beta$ -hydroxyacyl-CoA dehydratase YIHTD, the *Treponema denticola* trans-enoyl reductase TdTER or the endogenous truncated mitochondrial 2-enoyl thioester reductase Etr1 and the short-chain fatty acyl-CoA thioesterase CpFatB1 (Lian and Zhao, 2016, 2015). In **paper I**, we evaluated this design as well as own designs using the *S. cerevisiae* Fox2 and the *E. coli* thioesterase 'TesA for production of short chain free fatty acids. Unfortunately, we could not detect significant changes in free fatty acid production of the strains expressing any of the pathway designs compared to controls. The pathway was also tested with expression of an additional conversion step to fatty alcohols, which also did not show significant differences (data not shown).

Table 2. Thermodynamics of the reactions involved in the reverse  $\beta$ -oxidation pathway

Enzyme	Reaction <sup>1</sup>	$\Delta G^\circ$ (kJ mol <sup>-1</sup> )
Erg10/Fox3/YqeF/FadA	2 acetyl-CoA $\rightarrow$ acetoacetyl-CoA + CoA	26.7
Fox2	3-oxoacyl-CoA + NADH + H <sup>+</sup> $\rightarrow$ hydroxyacyl-CoA + NAD <sup>+</sup>	-19.9
Fox2	hydroxyacyl-CoA $\rightarrow$ <i>trans</i> -2-enoyl-CoA + H <sub>2</sub> O	2.1
tdTER	<i>trans</i> -2-enoyl-CoA + NADH + H <sup>+</sup> $\rightarrow$ acyl-CoA + NAD <sup>+</sup>	-57.9
'TesA	acyl-CoA + H <sub>2</sub> O $\rightarrow$ fatty acid + CoA + H <sup>+</sup>	-35.5
Pox1 (oxidative reaction) <sup>2</sup>	acyl-CoA + O <sub>2</sub> $\rightarrow$ <i>trans</i> -2-enoyl-CoA + H <sub>2</sub> O <sub>2</sub>	-42.6

<sup>1</sup> Reactions are presented and  $\Delta G^\circ$ 's were calculated in the synthetic (reductive) direction (except for POX1 reaction which is presented in the oxidative direction).

<sup>2</sup> The reaction for Pox1 is shown separately in the last column since Pox1 is not part of the reverse  $\beta$ -oxidation pathway and shown here to illustrate how it influences the thermodynamics of the  $\beta$ -oxidation pathway in native conditions.

We concluded that the reverse  $\beta$ -oxidation is an inefficient fatty acid production pathway when expressed in *S. cerevisiae*. This thought was supported by contact with other researchers working on this subject that also reported unsuccessful results in using this pathway in yeast for fatty acid production. This is also not surprising as there is a lack of literature describing use of this pathway successfully in eukaryotic organisms, while its use in *E. coli* cell factories continues to progress (Babu et al., 2015; Clomburg et al., 2015; Zhuang et al., 2014).

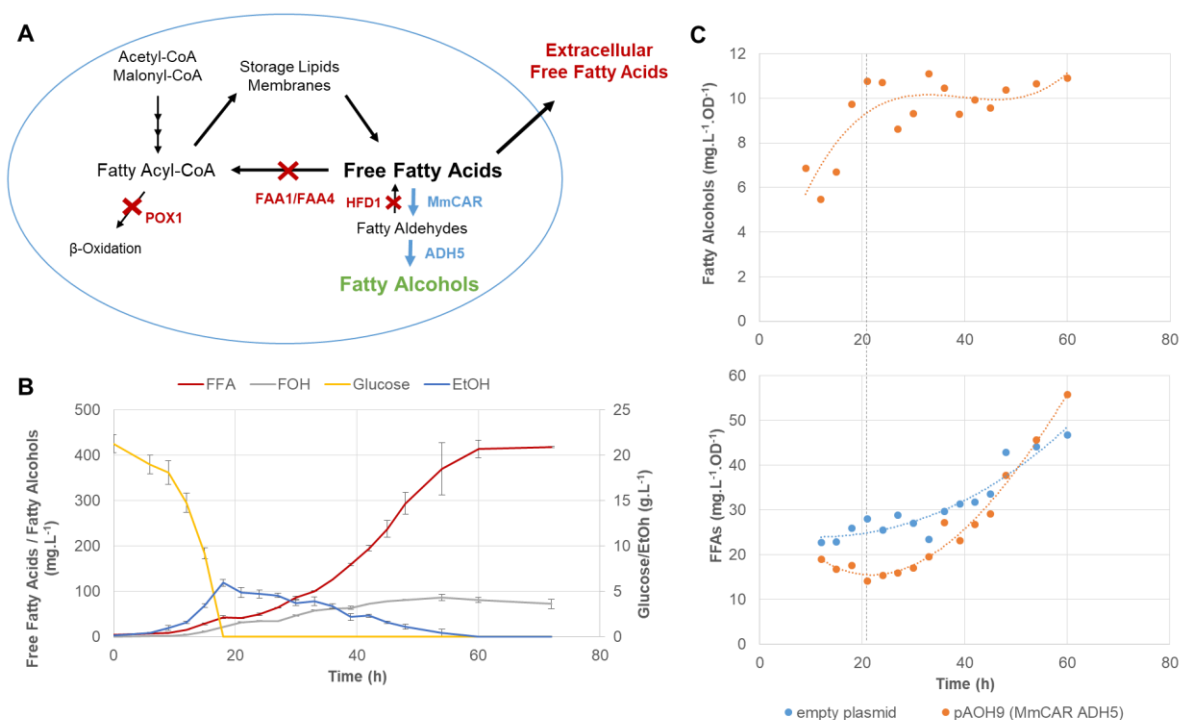
## Optimization of fatty acid pools for production of fatty acid-derived chemicals

Application of new strategies for overcoming challenges in fatty acid biosynthesis is one strategy for improving fatty acid biosynthesis of cell factories. However, the solution might not be in bypassing the FAS complex, but working with it. Regulation of the FAS machinery is partly dependent on downstream homeostasis mechanisms in controlling acyl-CoA and lipid pools (Faergeman and Knudsen, 1997; Kamiryo and Numa, 1973; Sumper and Träuble, 1973). Furthermore, understanding of fatty acid fluxes in the downstream lipid metabolism network is fundamental for production of oleochemical precursors that can be produced through conversion of specific lipid pools. Motivated by these aspects, we aimed to understand and work through engineering of downstream fatty acid metabolic fluxes in *S. cerevisiae* cell factories to enable more efficient engineering of these strains for producing either basic oleochemicals or oleochemical precursors.

The work here started with the need to understand the underlying fluxes behind a proof-of-concept fatty alcohol production strain regarding the pathways involved in the balance between acyl-CoA and FFAs and the conversion of both these pools towards fatty alcohols. Therefore, in **paper II** we first studied a *S. cerevisiae* strain previously designed for production of fatty alcohols from free fatty acids. In this strain YJZ08, *FAA1*, *FAA4* and *POX1* have been deleted, resulting in overproduction and secretion of FFAs to the extracellular medium as mentioned before. Using this background, a pathway for conversion of FFAs to fatty alcohols was expressed consisting of the carboxylic acid reductase gene from *Mycobacterium marinum* (*MmCAR*) (Akhtar et al., 2013) which converts FFAs to fatty aldehydes, and the endogenous alcohol dehydrogenase gene *ADH5* which in this pathway converts the formed fatty aldehyde to fatty alcohol (Zhou et al., 2016b) (**Figure 12A**).

We analyzed the production profile of this strain and realized that the FFA-to-biomass ratio constantly increases during the ethanol growth phase, while during the glucose phase it seems to decrease (**Figure 12B, C**). Furthermore, the strain that does not express the fatty alcohol pathway genes does not decrease in FFA/OD values during glucose phase and about 90% of produced fatty acids were produced during the ethanol growth phase (**Figure 12B**). This indicates that growth on ethanol seems to be favourable for FFA production compared to growth on glucose on a  $\Delta faa1 \Delta faa4 \Delta pox1$  background and conversion of FFA to fatty alcohol seems to be more efficient during growth on glucose. The fatty acid synthesis efficiency during ethanol is probably related to the availability of cytosolic acetyl-CoA needed for fatty acid biosynthesis, since growth on ethanol requires conversion of ethanol to acetate, which can then be converted to cytosolic acetyl-CoA through the acetyl-CoA synthases Acs1 and Acs2 (Chen et al., 2012). During this study, we also performed an experiment with glucose feeding through feed-beads, which allow for slow release of glucose to the medium, so cells can grow in a pseudo fed-batch condition of low glucose where pyruvate is imported to the mitochondria for respiratory growth, with no carbon flux towards acetate and ethanol. All samples taken during

this experiment showed no production of extracellular FFAs or fatty alcohols (data not shown), corroborating the hypothesis explored so far.



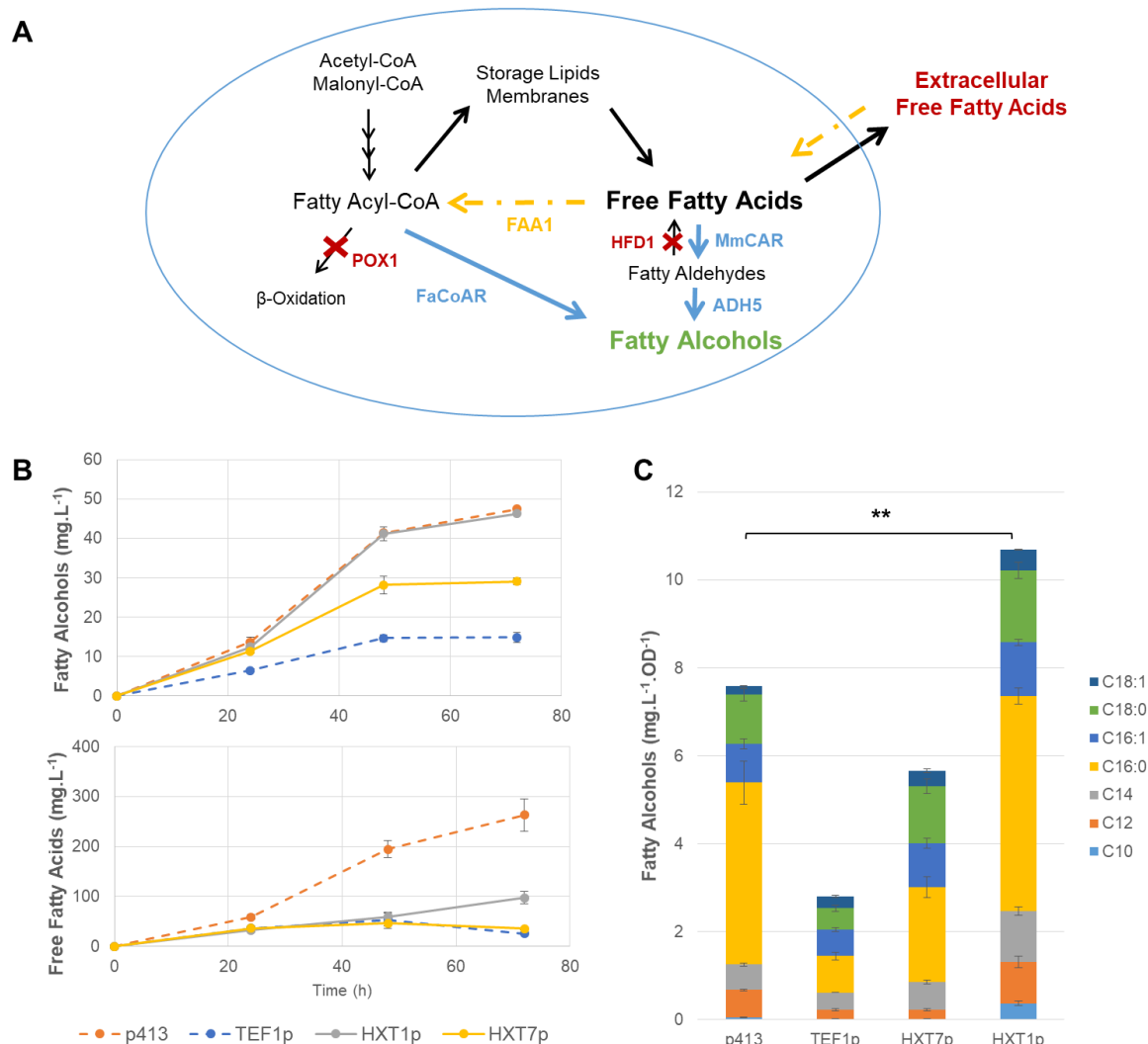
**Figure 12. Metabolic profiling of a fatty alcohol production platform strain.** A) Schematic representation of the fatty alcohol producing strain. *POX1*, *HFD1*, *FAA1* and *FAA4* have been deleted in this strain for FFA overproduction and *MmCAR* and *ADH5* are overexpressed for fatty alcohol production from FFAs. B) Total FFAs, fatty alcohols (FOH), glucose and ethanol (EtOH) levels in the culture of this strain. Samples were taken every 3 to 6 hours for 72 h. C) FFA and fatty alcohol titers normalized by the culture OD<sub>600</sub> at each time point comparing the fatty alcohol producing strain (orange line) and a control strain without the fatty alcohol production genes (blue line). Polynomial trendlines are presented for each set of points. Dashed line at 21h represents the point of diauxic shift where ethanol starts being used as a carbon source instead of glucose.

Although the fatty alcohol production pathway described benefits from high concentrations of substrate (FFA), the higher flux of FFA formation compared to its conversion to fatty aldehyde by *MmCAR* results in an overaccumulation of FFAs and consequential secretion to the extracellular medium. In this strain, only 20% of formed FFAs are converted to fatty alcohol, while most are secreted and not converted. In this paper, we design an alternate system where we fine tune the extent of FFA release through the disrupted fatty acid remodelling process by expressing *FAA1* under the control of different promoters that are either induced upon addition of Cu<sup>2+</sup> (*CUP1* promoter), induced at high glucose concentrations (*HXT1* promoter) or induced

at low glucose concentration (*HXT7* promoter). This was combined with simultaneous production of fatty alcohols both from FFAs and fatty acyl-CoA by expressing the MmCAR + ADH pathway plus a FAR gene (*FaCoAR*) from *M. aquaeolei* VT8 (**Figure 13A**). This design through fine-tuning of *FAA1* expression aimed for balancing of the two fatty acid pools and simultaneous conversion of both to fatty alcohols. Most analyzed strains had reduced levels of secreted FFAs due to presence of *Faa1* (**Figure 13B**), allowing for fatty acid re-import and activation mechanisms to come into play, but showed simultaneously high levels of fatty alcohols, proving that *Faa1* activity was low enough to still allow for high substrate availability. It also resulted in an increased specific flux towards fatty alcohol production, from which the best strain expressing *FAA1* under the *HXT1* promoter showed an increase of 30% in specific fatty alcohol accumulation (**Figure 13C**).

During this work, we have highlighted the importance of fatty acyl-CoA synthases such as *Faa1* for regulation of fatty acid levels. Higher expression of *FAA1* results in lower levels of FFAs and at the same time lower levels of total lipids and fatty acid-derived products, therefore pointing towards a direct relationship between *Faa1* activity, acyl-CoA levels and feedback regulation of fatty acid biosynthesis.

The results generated here allowed for an understanding of the extent to which acyl-CoA synthases contribute to regulating free fatty acid levels and consequently lipid remodeling processes. Studies so far have described this in the context of the presence or absence of these genes and the enzymatic properties of these acyl-CoA synthases. However, it was not obvious so far what effect these could have when expressed at different levels or culture times like it was studied here. Expression of *FAA1* with the lowest strength promoter we tested, *HXT1p*, still has a drastic effect in reducing secreted FFA. The effects observed in this paper reflects the importance and high activity of *Faa1*, and the extent to which it controls fatty acid levels and is fundamental for maintaining the homeostasis of fatty acid pools.

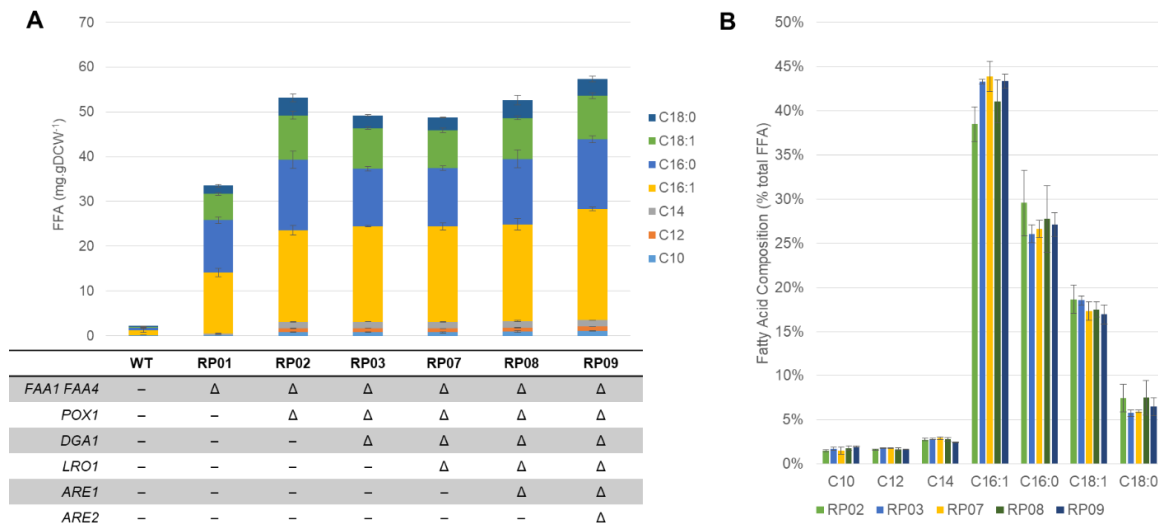


**Figure 13. Optimizing fatty acid and fatty alcohol fluxes through dynamic *FAA1* expression.** A) Schematic representation of strain YZFOH2 (YJZ08 pAOH9) expressing *FAA1* under different promoters. *POX1*, *HFD1*, *FAA1* and *FAA4* have been deleted in this strain and *MmCAR*, *ADH5* and *FaCoAR* are overexpressed from a 2 $\mu$  plasmid (pAOH9). B) Fatty alcohol and FFA titers produced by YZFOH2 expressing *FAA1* under control of the HXT1 or HXT7 promoters. For comparison, the same strain without expression of *FAA1* (p413) or with *FAA1* being expressed under control of the *TEF1* promoter were used. c) Final fatty alcohol titers at 72 h normalized to the total OD<sub>600</sub> values. Also shown is the distribution of fatty alcohols in terms of chain length and saturation levels. \*\*: p-value < 0.005 (Student's t-test).



## Dynamics of FFA turnover and its relationship with phospholipid metabolism

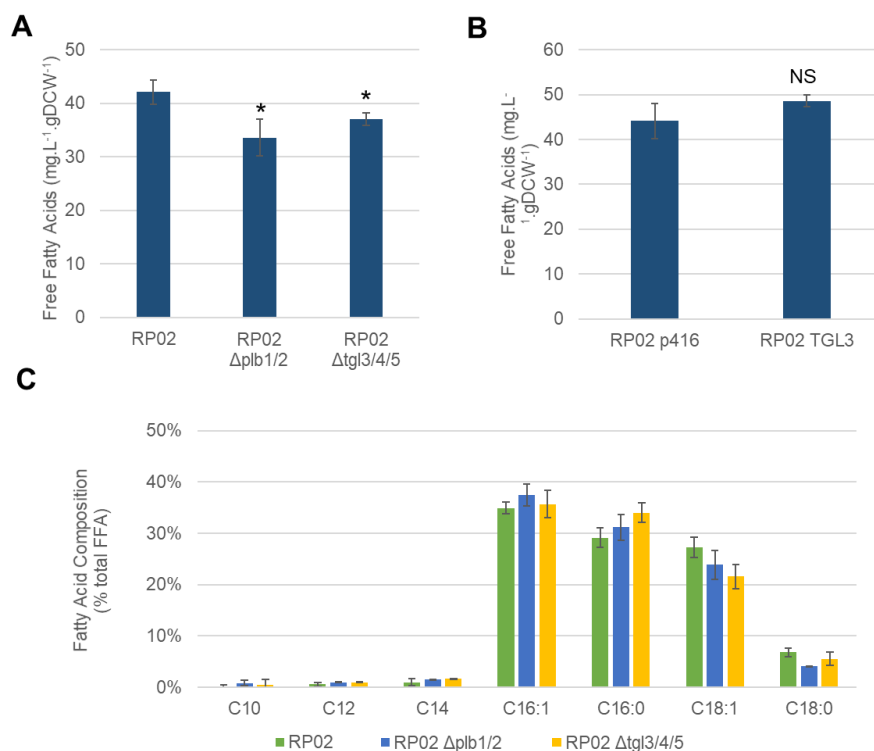
In order to engineer *S. cerevisiae* for high-level production of FFAs it is necessary to better understand the pathways and intermediaries involved in cytosolic FFA biogenesis as to which lipid pools and pathways are involved in that process. For that, we developed the work described in **paper III**. In this paper, we explored how different lipid pools contribute towards FFA synthesis through engineering of a  $\Delta faa1 \Delta faa4$  strain. Since *S. cerevisiae* does not have any cytosolic thioesterases to directly cleave acyl-CoA into FFAs, the origin of cytosolic and extracellular FFAs that are drastically increased upon deletion of *FAA1* and *FAA4* is an intriguing question. In this work, we first deleted *POX1* from the  $\Delta faa1 \Delta faa4$  strain in order to remove fatty acid oxidation on fatty acid levels, originating strain RP02 ( $\Delta faa1 \Delta faa4 \Delta pox1$ ). Subsequently, storage lipids were ablated from this strain by deletion of the genes in the TAG synthetic pathway *DGA1* and *LRO1* and removal of SE production pathway by deletion of the acyl-CoA:sterol acyltransferase genes *ARE1* and *ARE2*, originating strain RP09 ( $\Delta faa1 \Delta faa4 \Delta pox1 \Delta dga1 \Delta lro1 \Delta are1 \Delta are2$ ).



**Figure 14. Effect of storage lipids on FFA formation in a  $\Delta faa1 \Delta faa4 \Delta pox1$  strain.** A) Total (intra- and extracellular) FFAs were quantified for the different knockout strains lacking fatty acid re-activation (*FAA1* and *FAA4* deletions),  $\beta$ -oxidation (*POX1* deletion) and storage lipid formation (*DGA1*, *LRO1*, *ARE1* and *ARE2* deletion). B) Fatty acid distribution in the free fatty acid pool between the two strains as percentage of the total free fatty acids.

Removal of storage lipids did not present major differences in FFA levels since these were statistically similar between RP02 and RP09 (**Figure 14A**). Previous studies suggest that hydrolysis of storage lipids can be a source of cytosolic FFAs (Mora et al., 2012) and this was even explored as a FFA overproduction strategy by (Leber et al., 2015) through overexpression of the diacylglycerol synthase gene *DGA1* and the TAG lipase gene *TGL3*, leading to

production of up to 2 g/L of FFAs in rich media. In our study, even though storage lipids might play a role in FFA production when all lipid species are present, we first show these are not necessary and not the only source of FFA production. If storage lipids do contribute to the FFA pool, then this is compensated by some other lipid pool when these are ablated. However, fatty acid chain length and saturation levels of the FFA pool remain mostly unchanged between the strains, which can be an argument against this hypothesis (**Figure 14B**).

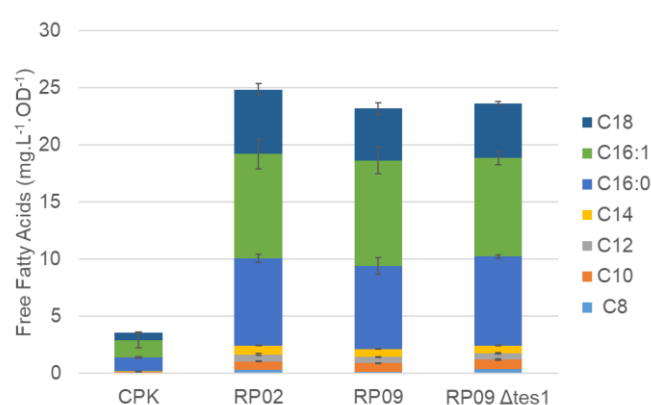


**Figure 15. Effect of deleting phospholipases or TAG lipases in a  $\Delta faa1 \Delta faa4 \Delta pox1$  strain.** A) Total (intra- and extracellular) FFAs quantified for RP02 ( $\Delta faa1 \Delta faa4 \Delta pox1$ ) and for RP02 with deletion of phospholipase genes PLB1 and PLB2 or TAG lipase genes TGL3, TGL4 and TGL5. B) Total FFA quantification of RP02 with an empty plasmid (p416) or a plasmid expressing the lipase TGL3. C) Fatty acid distribution in the free fatty acid pool between the two strains as percentage of the total free fatty acids.

In order to get additional insight into this matter, the main TAG lipase genes *TGL3*, *TGL4* and *TGL5* were also removed from the  $\Delta faa1 \Delta faa4 \Delta pox1$  strain but this only resulted in a 10% reduction in FFA levels (**Figure 15A**). Overexpression of the lipase gene *TGL3* did not bring significant changes to FFA production either (**Figure 15B**), as it has been observed before (Leber et al., 2015). Even though the results of lipase deletion from Mora et al., 2012 show a more drastic effect, both studies agree that neutral lipid hydrolysis is only part of the story when it comes to FFA production pathways and that removal of neutral lipids does not impair the FFA formation capacity of the cell and that other routes play this part when storage lipids

are removed. Furthermore, a previous study has linked TAG hydrolysis with phospholipid synthesis, with less phospholipid synthesis observed when a TAG lipase was deleted (Rajakumari et al., 2010). In fact, less phospholipids are observed in RP09, which supports this (Figure 17B, Paper III: Figure 3), and this evidence makes it difficult to isolate the effect of phospholipid hydrolysis when the level of this pool is affected by TAG hydrolysis.

At this point the question arises if the peroxisomal lipase *Tes1* could have an effect on this pathway through an indirect action involving peroxisomal transport of acyl-CoA or temporary localization to the cytoplasm. However, deletion of this gene did not result in a significant difference in FFA levels (Figure 16), eliminating this hypothesis.



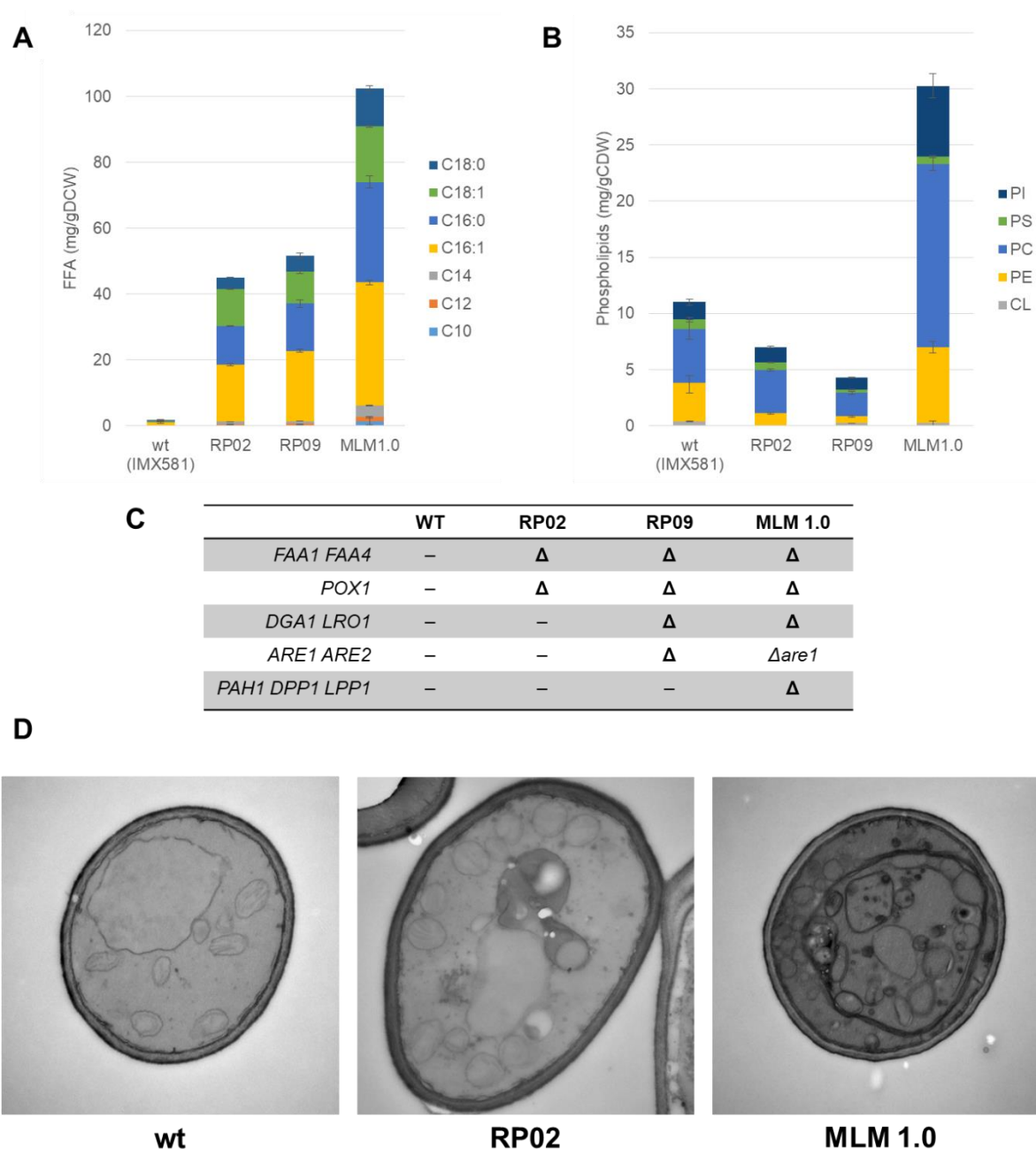
**Figure 16. Effect of *TES1* deletion on FFA formation.** A) Total FFAs quantified for RP02 (*Afaa1 Afaa4 Apox1*), RP09 (*Afaa1 Afaa4 Apox1*) and RP09 with deletion of the peroxisomal thioesterase gene *TES1*.

We further engineered RP09 in order to further focus the fatty acid fluxes towards phospholipid synthesis. For this, we removed the PA phosphatase genes *PAH1*, *LPP1* and *DPPI* generating strain MLM1.0. Deletion of these genes has been reported to eliminate formation of DAG and increase levels of PA (Chae et al., 2012; Han et al., 2007; Pascual et al., 2013), redirecting fatty acid flux towards the CDP-DAG phospholipid biosynthesis pathway. Enrichment in PA levels, as seen before, brings forward effects at the level of fatty acid and phospholipid biosynthesis regulation. High levels of PA increase the interaction strength of Opi1 with the ER membrane, therefore releasing inhibition of Ino2/Ino4-regulated genes (Henry et al., 2012; Kaadige and Lopes, 2006). The result from deletion of *PAH1*, *LPP1* and *DPPI* was a drastic increase of FFA levels by 98% and phospholipid levels by 8-fold (Figure 17A, B). Total fatty acids were also increased in this strain by 130% (Paper III, Supplementary Figure S5). These results highlight the relevance of the Ino2/Ino4/Opi1 system for control of the FAS machinery and phospholipid synthesis pathways, as reducing the effect of Opi1 repression leads to an increase of both total fatty acid content and phospholipid levels. As observed before in other studies

where PAH1 was deleted (Santos-Rosa et al., 2005), ER membrane expansion due to increased PA content was also observed in MLM1.0 (**Figure 17 D**)

MLM1.0 shows a simultaneous increase of both phospholipids and FFAs, therefore pointing towards a correlation between the two. This link was further investigated by deleting and overexpressing the main phospholipase B genes *PLB1* and *PLB2* responsible for hydrolysis of acyl chains from phospholipids in both positions *sn*-1 and *sn*-2. Deletion of *PLB1* and *PLB2* led to a drastic decrease of 46% in FFA levels and an increase of 105% in phospholipids (**Paper III: Figure 4**), which led us to pinpoint phospholipases as the main players in cytosolic and extracellular FFA biogenesis when fatty acid fluxes are redirected to phospholipids. Interestingly, deletion of these same phospholipase genes in the RP02 (*Δfaa1 Δfaa4 Δpox1*) strain led to a decrease of 20% in FFA levels (**Figure 15A**), indicating that these still have a role in FFA formation in this strain, but not as prominent as in MLM1.0. In fact, a previous study where phospholipase genes *PLB1*, *PLB2*, *PLB3* and *NTE1* were individually deleted from a *Δfaa1 Δfaa4* strain did not report a significant difference in FFA levels (Mora et al., 2012). A slight change in the fatty acid profile was also observed here, which was not observed in a significant way when the lipase genes were deleted, which supports this hypothesis (**Figure 15C**). This difference between the effect in MLM1.0 and RP02 might be simply due to a different effect of the phospholipases in question, since other phospholipases A and phospholipases B exist in *S. cerevisiae* and it is therefore possible that upon deletion of *PLB1* and *PLB2* other genes are upregulated to compensate for this lack of activity. We could not, however, successfully delete any additional phospholipases whenever we tried in MLM1.0, probably due to a toxic effect of phospholipid overaccumulation and/or inability to remodel this lipid pool. Another explanation for the significant remaining FFA formation in *Δfaa1 Δfaa4 Δpox1*, also supported by Mora et al., 2012, might be due to the presence of storage lipids that can provide a route for FFA synthesis when *PLB1* and *PLB2* are deleted.

Put together, our results in context of previous literature suggests a high flexibility of lipid metabolism in terms of lipid pool remodeling. Previous studies have shown a relationship between phospholipid and storage lipid synthesis (Carman and Henry, 1999; Mora et al., 2012), in which the carbon flux at the PA node can change to one pathway or the other according to factors such as growth phase and nutrient needs/supply (Fakas et al., 2011; Gaspar et al., 2011). This process is usually regulated by the PA phosphatase *Pah1* and the DAG kinase *Dgk1* (Gaspar et al., 2011; Han et al., 2008a, 2008b). It is therefore understandable that a major role of phospholipids in FFA formation is only observed when a high fatty acid flux is created and redirected to phospholipids, as phospholipids and storage lipids seem to complement each other for FFA supply in a *Δfaa1 Δfaa4* background. This phenomenon is probably tied to homeostasis and regulation of the fatty acid composition of the different lipid pools, since acyl chains in phospholipids need to be able to be rapidly cleaved and re-inserted in order to remodel membrane properties as required. For this, acyl chains not used in phospholipids are stored in neutral lipids, which also need to be able to be rapidly hydrolyzed in order to cope with the remodeling of the phospholipid fraction.



**Figure 17. Effect of deleting storage lipid formation and PA phosphatases on FFA and phospholipid levels.** A) Total FFA quantification of MLM 1.0 compared to strains RP09, RP02 and the control strain (wt). B) Phospholipid levels and composition in strains RP02, RP09 and MLM 1.0 compared to wt control strain. C) Summary table of strain phenotype. D) Transmission electronic microscope pictures of strains RP02 and MLM1.0 compared to wild-type.

## A better understanding of fatty acid dynamics

Throughout this section we studied physiological and metabolic outcomes of pathways and strategies currently employed in designing yeast cell factories for production of free fatty acids

and fatty acid-derived basic oleochemicals. More specifically, we studied the interconversion between the product of fatty acid biosynthesis, acyl-CoA, and its direct oleochemical precursor FFAs. We explored the pathways through which the acyl-CoAs can be converted into FFAs and which lipid pools are involved, and the dynamics and extent through which these FFA are reactivated through the action of acyl-CoA synthases.

We can understand from the studies here that acyl-CoA is the form through which fatty acids are transferred through different organelles, pathways and lipid pools. The presence of free fatty acids, on the other hand, seems to be often innocuous to most metabolic processes if not previously converted to their -CoA forms, since FFAs are not known substrates for any reaction in yeast apart from esterification with -CoA by acyl-CoA transferases. In terms of regulation, sensing of external fatty acid supply by the oleate-responsive element (ORE) (Gurvitz and Rottensteiner, 2006; Trzcinska-Danielewicz et al., 2008) has also been shown to depend on the presence of *Faa1-4* (Faergeman et al., 2001) which points towards the need of activation of fatty acids to acyl-CoA in order to transcriptionally regulate fatty acid-responsive genes.

By deleting *FAA1* and *FAA4* we enable overproduction and accumulation of FFAs partly because there is an inability by the cell to sense these increased levels and consequently feedback-regulate their synthesis. From this point on, increase in FFA synthesis will result in an increased accumulation of extracellular FFAs that do not create biosynthesis inhibition through feedback regulation.

An ability to achieve high titers of extracellular FFAs is extremely convenient for industrial processes since low solubility of hydrophobic products leads to their separation into a different phase, which facilitates downstream processing and purification.

We have therefore provided important knowledge that can undoubtedly be applied to design of *S. cerevisiae* cell factories for FFA production while exploring the advantageous properties of FFA deregulation and secretion.

## Part II: Improving Triacylglycerol Production

### Triacylglycerols - natural fat storage

Most eukaryotic organisms have evolved the same strategy for storing energy and carbon: neutral lipids. TAGs are neutral storage lipids and are often the main constituent of animal fat and plant oils. The same is true for many microorganisms such as oleaginous yeasts, which can accumulate up to 70% of their dry cell weight as lipids, mostly TAGs.

TAGs are the most common precursor in the oleochemical industry as explained before since these can be hydrolyzed into fatty acids or derivatized to other basic oleochemicals. But as we have evaluated before, direct production of FFA instead of TAGs becomes an advantage in this context. The important application it cannot replace is the use of specialty TAGs directly as food or cosmetic ingredients. TAGs with specific properties and defined fatty acid composition are of high value in the food and cosmetic market and some cases of synthetic TAGs produced through enzymatic processes have been registered in the past.

### Triacylglycerols production pathways in *S. cerevisiae*

TAGs are neutral lipids composed of a glycerol backbone in which all three hydroxyl groups have been esterified with fatty acids. The three positions available for fatty acids, referred to as *sn*-1, *sn*-2 and *sn*-3 are stereochemically different since the central carbon on position *sn*-2 is chiral. As described before, synthesis of TAGs in *S. cerevisiae* shares a common initiation pathway with phospholipid synthesis. Glycerol is first phosphorylated to glycerol-3-phosphate at the *sn*-3 position, which is then acylated with fatty acid chains at positions *sn*-1 and *sn*-2, in this order. Cleavage of the phosphate group by Pahl forms DAG with an hydroxyl group that can be acylated with the last fatty acid at the *sn*-3 position using acyl-CoA by Dgal or alternatively using the *sn*-2 chain of another phospholipid molecule by Lro1, resulting in production of TAGs. The fatty acid composition of the TAG pool is therefore dependent on the acyl-CoA produced by the fatty acid biosynthesis machinery and the specificity and preference of the acyltransferases that compose this metabolic pathway. As a result of this, each of the 3 positions on the TAG molecule will have different preference for the fatty acid present.

This complexity, dynamics and specificity of TAG synthesis is the main argument towards the use of *S. cerevisiae* for specialty TAG production. Through modulation of fatty acid synthesis and engineering of the TAG production pathway enzymes, it is possible to produce specialty

TAGs in *S. cerevisiae* with engineering ease and efficiency with which oleaginous yeasts and plants cannot compete. A clear case of this is the study by (Wei et al., 2017) where two different glycerol-3-phosphate acyltransferases (GPATs), two lysophosphatidate acyltransferases (LPATs) and two diacylglycerol acyltransferases (DGATs) from *Theobroma cacao* (cocoa tree) were expressed in different combinations in *S. cerevisiae* in order to produce TAGs with similar fatty acids and positioning as the ones found in cocoa butter. This had the effect of increasing the content of cocoa butter-like lipids by up to 2.3-fold (Wei et al., 2017).

In order to use this advantage for production of specialty TAGs, it is necessary to first transform *S. cerevisiae* into a high TAG producing organism and develop its full potential for accumulation of this lipid species. As such, previous strategies have been applied to increase the metabolic flux through the TAG synthesis pathway. A previous study (Kamisaka et al., 2007) increased lipid content in *S. cerevisiae* to 30% producing 0.44 g/L of TAGs from glucose by deleting the transcription factor gene *SNF2*, which has been previously identified as a regulator of lipid metabolism with an unclear mechanism (Kamisaka et al., 2006), and overexpressing *DGAI1*. A follow-up study expressed a truncated version of *DGAI1* in a *Δdga1* strain reaching up to 50% lipid content in a 10% glucose culture with a production titer of 0.97 g/L of lipids (Kamisaka et al., 2013). A different study used overexpression of *ACC1*, *FAS1* and *FAS2* under a constitutive strong *TEF1* promoter to obtain a strain with 17% lipid content and 0.17 g/L of lipids from 2% glucose (Runguphan and Keasling, 2014). Another strategy reconstituted an algal TAG production pathway through expression of a DGAT from *Chlamydomonas reinhardtii* (*CrDGTT2*) and overexpression of the endogenous *PAH1* together with deletion of the DAG kinase gene *DGK1* and the phospholipid biosynthesis gene *OPI3* (Hung et al., 2016) reaching 12% of lipid content and 0.17 g/L of TAG titers.

TAG was also produced using glycerol as the sole carbon source. Overexpression of the glycerol kinase gene *GUT1* together with *DGAI1* and *LRO1* resulted in production 8% TAG content and a titer of 0.02 g/L of TAG from a 2% glycerol medium (Yu et al., 2013).

Even though these strategies led to *S. cerevisiae* strains capable of accumulating high amounts of their dry cell weight as lipids, conversion yields of glucose to TAGs only reached a maximum of 0.047 gTAGs/gGlucose. Assuming the maximum theoretical yield for production of TAGs through the metabolic network of *S. cerevisiae* to be approximately 0.32 g/g (Caspeta and Nielsen, 2013), these strategies achieved values that are still 14% of the maximum yield possible in *S. cerevisiae* for production of TAGs, leaving room for improvement into transforming this yeast into a high TAG producer.

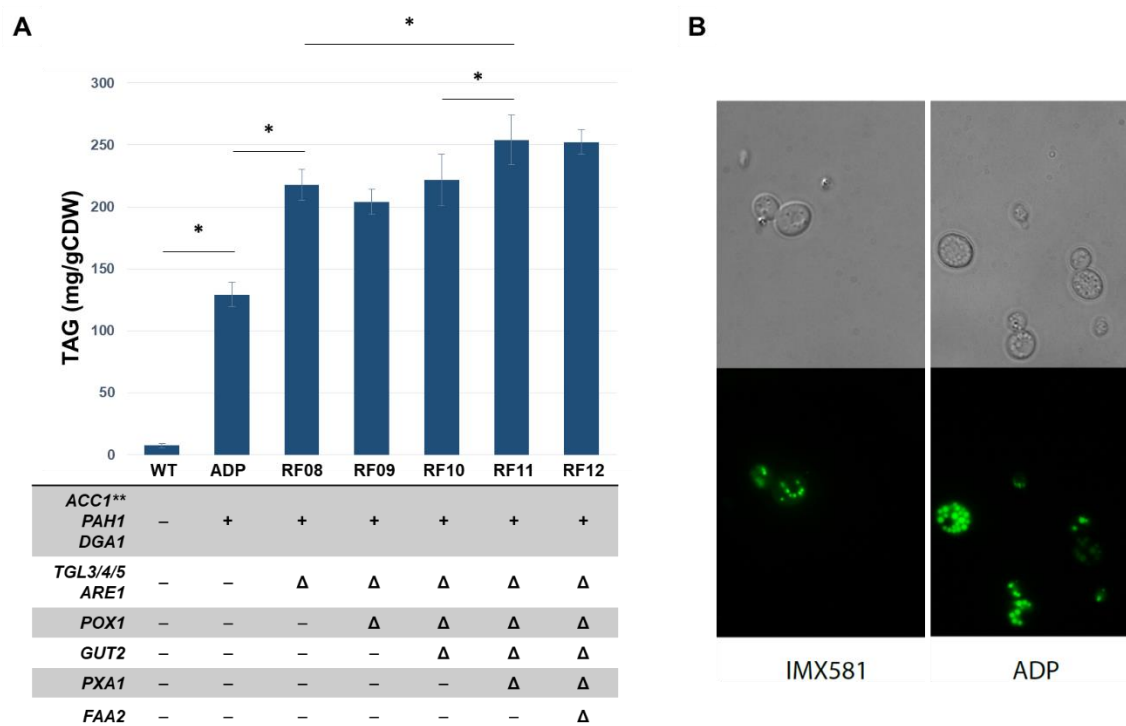


## Metabolic engineering of *S. cerevisiae* for high-level TAG production

Although different strategies have been applied to engineer *S. cerevisiae* for accumulation of high contents of TAGs, strategies are still disperse and have only targeted a few genes and pathway steps. With the advent of faster genome engineering tools like CRISPR-Cas9, it is possible to generate heavily engineered strains faster and more efficiently. Based on previously validated approaches, in **paper IV** we have developed *S. cerevisiae* strains that were able to carry high flux through fatty acid biosynthesis and TAG production pathways.

The first major step for successful application of this strategy was the use of an *ACC1* mutant allele in which two identified phosphorylation sites have been mutated in order to express a constitutively de-regulated form. The Ser1157 and Ser659 residues of Acc1 have been identified as potential phosphorylation sites for Snf1, either through phosphoproteome analysis for Ser1157 (Ficarro et al., 2002) or through bioinformatics analysis for Ser659 using the conserved phosphorylation recognition motif for Snf1 (Dale et al., 1995). Mutation of these two serines to alanine residues highly increased activity of Acc1 *in vivo* by 2.5-fold and consequently increased production of malonyl-CoA and fatty acyl-CoA derived products by more than 3-fold (Shi et al., 2014). In our study, we first expressed this *ACC1* double mutant (*ACC1\*\**) together with an extra chromosomal copy of the endogenous *PAH1* and *DGA1* expressed under control of the strong constitutive promoters *PGK1p* and *TEF1p*, respectively. The resulting strain, ADP, was able to produce up to 13% of its dry weight content as TAGs in 2% glucose minimal medium, a higher than 10-fold increase in comparison to the reference wild-type strain and showed a clear increase in LD accumulation when stained with BODIPY (**Figure 18**).

The second major step in TAG improvement was the deletion of the TAG lipase genes *TGL3*, *TGL4* and *TGL5* with the goal of removing TAG hydrolysis and avoiding TAG mobilization. At the same time, we aimed to remove the two acyl-CoA:sterol acyltransferase genes *ARE1* and *ARE2* in order to focus neutral lipid synthesis into TAGs instead of SEs, but technical difficulties did not allow a successful removal of *ARE2*. The new strain RF08 (*ACC1\*\* PAH1 DGA1 Δtgl3 Δtgl4 Δtgl5 Δare1*) showed a 68% increase in TAGs levels compared to strain ADP, with a final TAG content of 22% of its dry cell weight (**Figure 18A**). Further engineering aimed at removing the  $\beta$ -oxidation gene *POX1* and the gene coding a subunit of the peroxisomal transporter involved in the import of acyl-CoA *PXA1* with the goal of removing transport and oxidation of fatty acids in the peroxisome. The glycerol-3-phosphate utilization pathway was also targeted by deleting the glycerol-3-phosphate dehydrogenase encoding gene *GUT2* with the objective of increasing the supply of glycerol-3-phosphate for the TAG synthesis pathway. The strain RF11 with all these modifications was able to produce up to 25% of its dry weight as TAGs with a production titer of 1.76 g/L from 20 g/L of glucose, which translates to 27.4% of the maximum theoretical yield of TAG production from glucose.



**Figure 18. Effects of metabolic engineering of *S. cerevisiae* for high TAG production.** A) TAG quantification of different engineered strains. TAGs were quantified for strains with the implemented push-and-pull approach (*ACC1\*\**, *DGA1* and *PAH1* overexpression) and lacking TAG lipases (*TGL3/4/5* deletion), sterol acyltransferases (*ARE1* deletion),  $\beta$ -oxidation (*POX1* deletion), glycerol utilization (*GUT2* deletion), the acyl-CoA peroxisomal transporter (*PXA1* deletion) and fatty acyl-CoA synthetase (*FAA2* deletion). Strains were grown for 72h in minimal medium containing 2% glucose. \*p-value < 0.05 (Student's T-Test: two-tailed, two-sample equal variance). B) Lipid droplet staining of strains IMX581 and ADP with BODIPY and analyzed with a fluorescent microscope showing lipid droplets in green.

The results from TAG production yields from glucose in this paper are impressive in a way that represent a 160% increase over the previous benchmark strain for TAG production in *S. cerevisiae* (Kamisaka et al., 2013). This is the result of a successful push-and-pull strategy where many factors were taken into account, from the upregulation of the fatty acid biosynthesis pathway through *ACC1\*\** overexpression, to upregulation of the TAG synthesis pathway from PA, supply of glycerol-3-phosphate as a substrate for the pathway initiation, removal of competing fluxes for SE formation, removal of TAG hydrolysis enzymes and deletion of genes involved in fatty acid oxidation.

Even though the lipid content percentage for the obtained strains in this study is much lower than the maximum reported for *S. cerevisiae*, the biomass values obtained are much higher than in all the other studies here analyzed in the context of TAG production. The reasons for this might vary and it might be strongly related to the culture media used in each study. While here we used minimal media buffered with  $\text{KH}_2\text{PO}_4$ , many of the studies reported used variants of

SD media, which first are sensitive to pH variation due to low buffer capacity and secondly have different nitrogen bases, amino acid content and consequently a different C/N ratio. All these factors will affect biomass yield and lipid metabolism and the correct balance is required for an efficient production of the product in question.

## Improving TAG production and accumulation through the use of perilipins

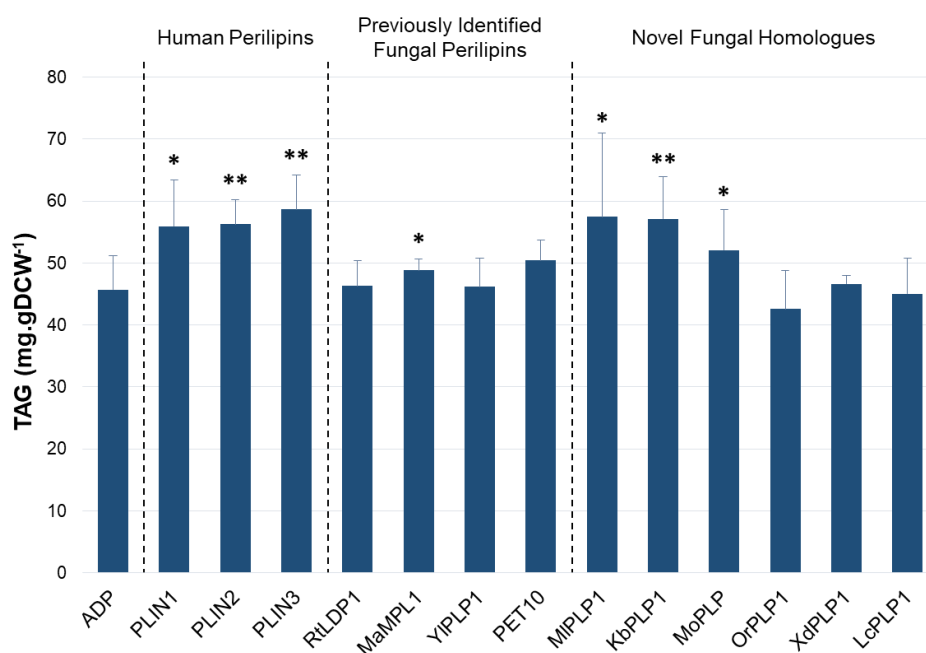
The work developed in **paper IV** allowed us to generate *S. cerevisiae* strains capable of accumulating TAG amounts that were more than 20-fold higher than a wild-type strain, in fact turning this yeast into an oleaginous one by definition. The first modifications consisting of *ACC1\*\**, *PAH1* and *DGAI* overexpression proved to be the most relevant ones in terms of improvement, followed by deletion of the lipase genes. Modifications from that point onwards only resulted in small improvements of the TAG levels, providing the feeling that a plateau of maximum productivity was being reached. Contemplating this idea, we realized that increasing the fluxes towards TAG production provokes drastic increase of LD distribution and number in the cell, and envisioned that structural limitations of the LD assembly and stabilization in the context of the cell might be hindering further development.

**Paper V** describes the work where we focused on engineering the LD at the level of its biogenesis mechanisms and protein-mediated stability in the cell. LD biogenesis and assembly mechanisms have been explained in a previous section and as we have seen, besides enzymes involved in synthesis of storage lipids necessary for LD biogenesis, there are 3 main families of proteins involved in structural aspects of the assembly mechanism: Perilipins, FIT proteins and seipins.

The first part of **paper V** deals with aspects of perilipins. Perilipins are a family of PAT proteins that can have different roles in LD biogenesis and modulation of lipid hydrolysis through lipases and autophagy (Sztalryd and Brasaemle, 2017). When the project started, no perilipins had been described in *S. cerevisiae*, it was only during the development of the work that the gene *PET10* was shown to code for a *S. cerevisiae* perilipin protein (Gao et al., 2017). Pet10 was shown to bind early to nascent LDs, and biogenesis rate is decreased in *pet10Δ*. Moreover, LDs isolated from *pet10Δ* are fragile, aggregate and fuse *in vitro*, showing a role of this perilipin in maintaining LD stability. Pet10 was also shown to genetically interact with seipin and Fit2 and modulate the activity of Dga1 (Gao et al., 2017).

Even though a perilipin is present in this yeast, a previous study has reported improvements in TAG synthesis rate, reduced TAG hydrolysis and promoted LD formation when human perilipins genes *PLIN1*, *PLIN2* and *PLIN3* as well as the Oleosin 1 from *Arabidopsis thaliana* were expressed in yeast (Jacquier et al., 2013). Based on this, we speculated that strains with increased TAG accumulation and therefore enlarged LD structures would benefit from perilipin

expression and as such expressed the same human perilipin genes PLIN1-3 described before (Jacquier et al., 2013) in the strain ADP that was developed during **paper IV**. The reason to evaluate the genes in this strain was that further engineered strains had deletions of the main lipase genes, and thus it would not be possible to detect effects arising from inhibition of lipase activity. Expression of PLIN1-3 improved the production of TAG in the ADP strain by 22%, 23% or 28% respectively (**Figure 19**), validating our hypothesis that expression of these proteins would be beneficial.



**Figure 19. Effect of expressing different perilipin proteins in the TAG levels of the ADP strain.** Different perilipin homologues were expressed in a previously engineered strain ADP overexpressing *ACCI*\*\*, *PAHI* and *DGAI* for accumulation of high TAG levels. Three different categories are represented: Human perilipins: *PLIN1*, *PLIN2/ADRP* and *PLIN3/TIP47*; Fungal perilipin-like protein homologues identified in the literature: *RtLDP1* from *Rhodospiridium toruloides*, *MaMPL* from *Metarhizium anisopliae*, *YlPLP1* from *Yarrowia lipolytica* and *Pet10* from *Saccharomyces cerevisiae*; and 6 novel candidates identified from a bioinformatic analysis using BLASTP: *MIPLP1* from *Melampsora larici-populina* 98AG31, *KbPLP1* from *Kalmanozyma brasiliensis*, *MoPLP1* from *Mixia osmundae* IAM 14324, *OrPLP* from *Obba rivulosa*, *XdPLP1* from *Xanthophyllomyces dendrorhous* and *LcPLP1* from *Leucosporidium creatinivorum*. Resulting strains were cultivated in minimal medium with 2% glucose for 72 h. \*p-value < 0.05, \*\*p-value < 0.005 (Student's t test, one-tailed, unequal variance).

Continuing the study on using perilipins for improving TAG production, we aimed to screen for fungal homologues of perilipin proteins to evaluate the potential of perilipins that would increase this effect more efficiently than the human perilipins. As such, we started from previously identified genes from oleaginous yeasts such as *R. toruloides* or *Y. lipolytica* (Zhu

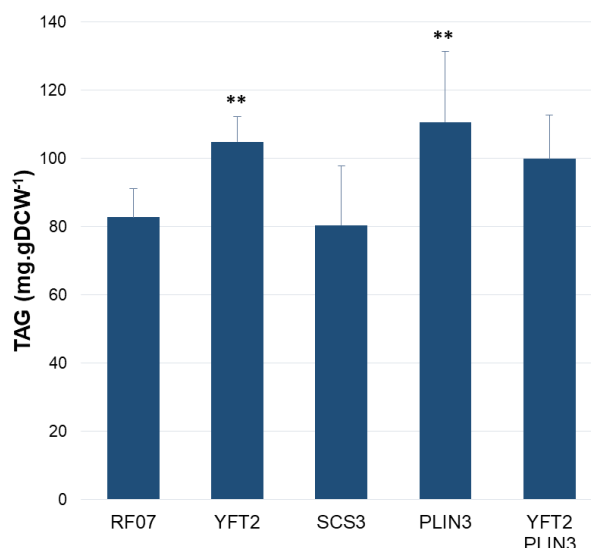
et al., 2015) and expanded the bioinformatics search using BLASTP, selecting 6 additional genes with different sequence similarity values. All genes were expressed in strain ADP, but the highest increases registered were after expression of 2 genes from *Melampsora larici-populina* (*MIPLP1*) and *Kalmanozyma brasiliensis* (*KbPLP1*) which improved the TAG content by 25% and 26%, respectively, still lower than the improvement for *PLIN3* (**Figure 19**) Furthermore, the beneficial effect from *PLIN3* was independent from lipase action, and it showed an even better improvement, by 34%, when expressed in RF07 (*ACC1\*\* PAH1 DGAI  $\Delta$ tg13  $\Delta$ tg14  $\Delta$ tg15  $\Delta$ are1*). The effect from the fungal perilipins *MIPLP1* and *KbPLP1* seemed to be more related to the lipase effect since its improvement went down to 22% and 9% respectively when expressed in RF07 (**Paper IV: Figure 3**).

Since *Plin3* in humans was described as having a strong impact on the LD biogenesis process (Bulankina et al., 2009), it had been shown to promote LD budding in yeast (Jacquier et al., 2013), and our data showed an improvement independent of lipase effect, we hypothesized that the observed improvement in TAG production by *Plin3* expression was due to a stimulation of the LD biogenesis and assembly process.

## Enhancing lipid droplet biogenesis for improved TAG accumulation

### The effect of *FIT2* proteins in increasing TAG levels

To explore the potential of improving the LD biogenesis effect as an engineering strategy, we overexpressed the two *FIT2* gene homologues that can be found in the *S. cerevisiae* genome, *YFT2* and *SCS3*, in RF07. Surprisingly, overexpression of *YFT2* improved TAG accumulation levels by 27% but the same was not true for *SCS3*, which did not improve TAG accumulation (**Figure 20**). Even though *SCS3* and *YFT2* are homologues (Choudhary et al., 2015), the two have been previously shown to have different genetic interaction dynamics and therefore different functions (Moir et al., 2012). Although it is not clear which different factors could be in play that explain the difference observed here, it was shown that *SCS3* is more involved in promoting phospholipid synthesis in reaction to ER stress and to positively interact with the transcription of *DGK1* and some phospholipid biosynthetic genes, whereas *YFT2* was neutral in this sense (Moir et al., 2012), which could partially explain the negative effect from *SCS3* overexpression in TAG synthesis.



**Figure 20. Effect of FIT proteins on TAG accumulation in RF07.** A) Endogenous FIT protein genes YFT2 or SCS3, and/or human perilipin gene PLIN3 were expressed in RF07 (ADP  $\Delta$ tg13/4/5). Resulting strains were cultivated in minimal medium with 2% glucose for 72h. \*p-value < 0.05, \*\*p-value < 0.005, NS: not statistically significant (Student's t test, one-tailed, unequal variance).

Observation of LDs stained with fluorescent dyes through fluorescent microscopy did not show any apparent differences in LD number or morphology upon expression of either perilipins or the FIT proteins. This observation however, is hindered by the fact that RF07 contains a large number of LDs for each cell, therefore making it difficult to detect any increase in this number. Confocal microscopy associated with imaging processing software could possibly provide more insight on this through quantitative data on the number of LDs per cell.

Overall, overexpression of FIT proteins has been shown to stimulate LD formation and create fat phenotypes in other organisms (Cai et al., 2017; Tan et al., 2014), which is in accordance to what we observed in our work for *YFT2* expression. The non-concordant result for *SCS3* overexpression indicates a functional difference between the two genes. While Yft2 might be more actively involved with the structural aspects of LD biogenesis, the role of Scs3 might be majorly towards regulating phospholipid metabolism and response to Inositol-mediated regulation, as it was also observed by (Moir et al., 2012).

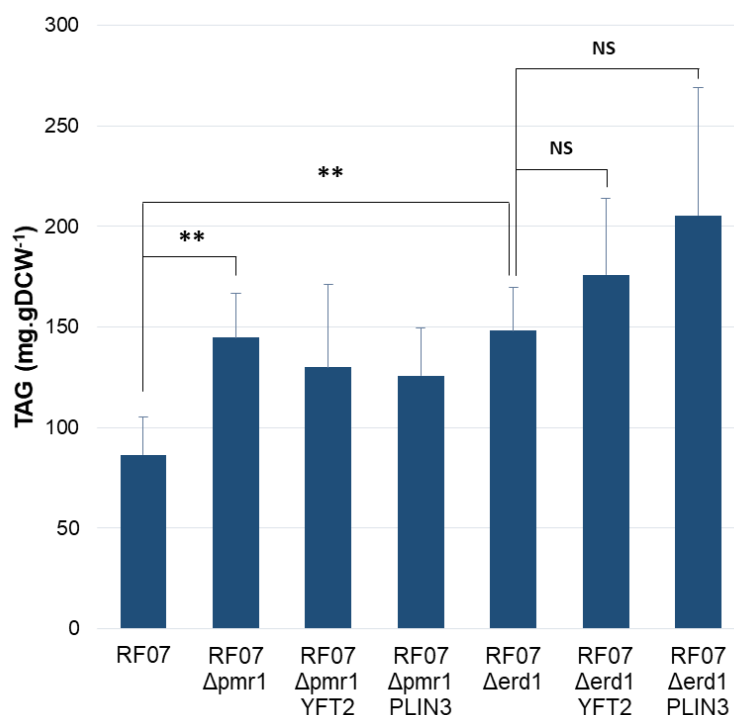
We did not observe a cumulative effect from simultaneous overexpression of *PLIN3* and *YFT2*. The reason for this is elusive since the exact mechanism for the effect of each protein is not well described. However, we can assume that both act on LD biogenesis, even if through different mechanisms, and probably expression of either one stimulates the whole LD assembly mechanism sufficiently so that additional expression of another factor would not further enhance it.

## Increased TAG levels through stimulation of ER stress

Through looking at the correlation between improved TAG synthesis and enhanced LD biogenesis, we realized that conditions that cause ER stress have been strongly associated with increased LD formation rate and higher TAG and SE content in *S. cerevisiae* (Fei et al., 2009; Moir et al., 2012). Stress responses are an output of intracellular monitoring systems that deploy a set of regulatory pathways to respond to cellular imbalances. One of the best characterized pathways is the unfolded protein response (UPR) pathway, which monitors ER homeostasis and responds to alleviate effects of ER stress (Wu et al., 2014). In yeast, UPR relies exclusively on the Ire1 (inositol-requiring enzyme-1) pathway, which activates a multitude of genes including genes involved in lipid metabolism, specifically inositol-regulated phospholipid genes (Jesch et al., 2006, 2005). The UPR is therefore a pathway for maintaining lipid homeostasis in the ER membrane and can control lipid synthesis genes in order to balance membrane lipid composition (So et al., 2012) as well as playing a fundamental role in ER membrane expansion (Schuck et al., 2009).

Interestingly, various gene deletions causing defects in protein glycosylation or ER-associated protein degradation (ERAD) lead to an ER stress response (Chantret et al., 2011; Uchimura et al., 2005) and have been associated with an increased number of LDs in the cell as well as an increase in TAG and SE content (Fei et al., 2009). This outcome, however, was independent of Ire1 and consequently the UPR since  $\Delta ire1$  strains still had the same increase in neutral lipid content and increased LD number. The effect was also observed without higher levels of Dga1, Lro1, Are1 and Are2 in the cell extracts, indicating an independence of upregulating any storage lipid synthesis.

To evaluate the potential effect of this, we individually deleted in strain RF07 two of these identified genes, *ERD1* encoding for a predicted membrane protein required for lumenal ER protein retention, and *PMR1* encoding for a  $\text{Ca}^{2+}/\text{Mn}^{2+}$  P-type ATPase. The deletion phenotypes of these genes have been previously associated with promoting ER stress and an increase in TAG content and LD number (Fei et al., 2009). In our study, it resulted in an increase of 72% and 67% in TAG content when *ERD1* or *PMR1*, respectively, were deleted. We additionally then expressed either *YFT2* or *PLIN3* in the  $\Delta erd1$  strain, which increased the TAG content by 104% and 138% respectively compared to RF07. Even though expression of either *YFT2* or *PLIN3* showed an average additive effect with the *ERD1* deletion, there was a large clone variability for these strains, which did not allow for a statistically significant result (**Figure 21**). Speculating, expression of genes from a plasmid or from the *TEF1* promoter might be compromised in a  $\Delta erd1$  phenotype, leading to inconsistency between transcription efficiency in different clones. Other than that, it is possible that overexpression of proteins that interact with the ER in this phenotype create instability in LD assembly. Expression assays using integrated gene constructs with promoters of different strengths can probably help solve this issue and allow for further clone consistency.



**Figure 21. Effect of promoting ER stress and FIT protein overexpression on TAG accumulation in RF07.** B) Genes ERD1 and PMR1 involved in ER stress were deleted. Additional expression of YFT2 or PLIN3 was also evaluated in these deletion strains. Resulting strains were cultivated in minimal medium with 2% glucose for 72h. \*p-value < 0.05, \*\*p-value < 0.005, NS: not statistically significant (Student's t test, one-tailed, unequal variance).

From an engineering perspective, enhancement of TAG levels through ER stress stimulation and enhancement of LD biogenesis through either Yft2 or Plin3 was a success, with high titers reported (**Figure 21**) and low penalty to growth (**Paper V: Supplementary Figure S2**). The mechanism of action concerning TAG and LD increase through ER stress caused by these deletions is so far unknown. The data generated here are however supportive of an Ire1-mediated effect, since *SCS3* expression, but not *YFT2*, has been shown to be related to and affected by Ire1 activity (Moir et al., 2012).

We have here reproduced the observation of ER stress sensing mechanisms related to lipid homeostasis that are UPR-independent, pointing towards the existence of mechanisms that still need to be further understood. Other studies have also suggested and reported UPR-independent regulation of ER stress related to reorganization and morphology of the ER membrane, but without clear evidence on the molecular mechanisms of these (Sano and Reed, 2013; Schuck et al., 2009; Varadarajan et al., 2012)



## Engineering approaches for lipid accumulation must go beyond metabolism

A strong link was shown during our work between stimulation of LD assembly and accumulated TAG levels. Also, as shown before (Schuck et al., 2009), there seems to be a crosstalk between lipid homeostasis, ER stress response, ER membrane expansion and phospholipid-TAG interconversion pathways.

Increasing metabolic fluxes towards neutral lipid formation resulting in more than 25-fold increase of TAG levels will bring consequences to the cell in terms of its lipid homeostasis and activate responses to counterbalance that effect. Since TAG synthesis is partly located in the ER and partly on the LD, increasing the flux of this pathway will create a pressure on the ER in which this needs to increase LD assembly rates in order to properly pack these neutral lipids and avoid ER deformities from excess neutral lipid accumulation. Native mechanisms probably exist to upregulate this process to some extent, and as such we can observe an increase in LD number in the cell in this situation. However, it is only natural that the evolution process of *S. cerevisiae* did not adapt this organism for such an increase in TAG synthesis pathways. In this sense, increasing the protein number of LD assembly factors can help this process by increasing the number of LD formation sites. In the same way, directly promoting a strong ER stress response will also result in an increased rate of LD biogenesis to levels more beneficial than the upregulation caused by a sensing of increased TAG levels.

The results from this section provide additional insight on the links between TAG synthesis, LD assembly and ER stress. Furthermore, the study from **paper V** is a new approach to engineering of cell factories for TAG synthesis, relying on structural factors of organelle biogenesis instead of a pure metabolic engineering approach.

The success of this approach clearly shows the need to consider structural, physiological and regulatory factors in cell factory design. When engineering lipid metabolism it is of most importance to consider the cellular context that supports this complex network and consider the subcellular localization and molecular structures in which these reactions occur.



## Conclusions and Perspectives

The work developed during this thesis provided new advancements and knowledge for metabolic engineering of *S. cerevisiae* cell factories with regard to lipid and fatty acid-derived products.

In Part One of this thesis, the reversed  $\beta$ -oxidation was evaluated as a potential pathway for production of fatty acids in yeast as a more efficient alternative to fatty acid biosynthesis, but with no success. From that, the focus shifted towards fatty acid dynamics in the downstream lipid metabolism network. Fine-tuning of *FAA1* expression on a fatty alcohol production platform demonstrated the high relevance of acyl-CoA synthases in lipid homeostasis processes. Following up on the subject, the interest was then on the pathways leading to cytosolic free fatty acid formation when long chain acyl-CoA synthases are absent. This was done through engineering of a new strain with a constrained lipid metabolism network that focused on phospholipid accumulation and consequently free fatty acid production from phospholipid hydrolysis.

From the three studies put together, one can generally conclude that lipid remodeling is a very dynamic process that involves balanced hydrolysis of storage lipids and phospholipids, and that the process is highly regulated and flexible in a way that is self-compensatory to keep cellular homeostasis of fatty acid pools. Deletion of the *FAA1* and *FAA4* genes causes over 50-fold increase in FFA levels, going from levels that composed around 0.1% of the cell weight to more than 5%, a percentage which is almost double the total amount of lipids present in a wild-type cell. These results reveal the high fatty acid turnover rates in these remodeling processes, which shows not only the potential for this organism to produce high amounts of fatty acids, but also how tight the regulation mechanisms controlling these are. This regulated high flux of lipid remodeling is probably tied to the natural evolution of this species, which focused on adaptability and robustness, one of the reasons which make this yeast so desirable in industry. The ability to quickly remodel lipids is an advantage for fast adaptation to new environments and stresses.

The strains generated in **paper III** represent interesting platforms for further development of cell factories. The strains with the constrained lipid metabolism network (MLM1.0 and variants) are first of all interesting due to their PA-mediated upregulated fatty acid metabolism. The upregulation of the UAS<sub>INO</sub>-regulated gene set with a linear fatty acid metabolism towards phospholipids represents an interesting platform for any product that can be produced from intermediates in this pathway. These can further be useful for screening of gene variants that catalyze one of the steps of this pathway, since the linear flux allows for a straightforward screen of the metabolic output. When it comes to production of free fatty acids, this strain is

compatible with strategies developed in other studies. Without the use of gene overexpression cassettes or markers, the strain is genetically compatible with further engineering without problems that might come from presence of genome editing marks. This strain can be further engineered with other aspects seen through this work, such as enhancement of cytosolic acetyl-CoA supply, expression of *Acc1* mutants, thioesterases or other FFA or acyl-CoA consuming pathways.

In Part Two, we contributed major advancements in the development of *S. cerevisiae* strains for TAG production. This was achieved not only by using metabolic engineering approaches, but by bringing into play enhancement of the LD assembly mechanism. Since the mechanisms of LD biogenesis and the role of the factors involved are not completely described or understood, our approach relied more on screening and trial-and-error. However, the results generated here become very valuable exactly due to this lack of knowledge. By using the strains built in **paper IV**, we could study the mechanism of LD formation in a model where the synthesis of TAG is largely increased. Since these mechanisms are very conserved among eukaryotes, this could provide valuable information for understanding obesity phenotypes in mammals.

A particularity about **paper V** was that the TAG levels quantified for the ADP and RF07 strains carrying empty plasmids were consistently much lower than the quantification of the same strains grown with uracil supplementation on **paper IV**. As a consequence, in **paper V** we did increase by almost 140% the levels of RF07, but this was compared to the control strain with the empty plasmid and as such in **paper V** we did not obtain levels as high as **paper IV**. However, if this actually is an effect of plasmid presence, then further genome engineering of the best strains from **paper IV**, such as RF11, with the elements from **paper V** such as deletion of *ERD1* and expression of *YFT2* or *PLIN3*, could increase the TAG levels in these strains to even higher values, potentially reaching values as high as 50% of cell dry biomass as TAGs and production above 50% of the maximum theoretical yield. So again, here it was developed different compatible strategies that contribute to the advancement of the technology and can be combined to achieve levels close to industrial applications.

The observational conclusion from all the work put together here is that engineering of lipid metabolism is a problem more complex than just a simple network of chemical reactions mediated by enzymes. Considerations regarding regulation networks, cellular context, localization and processes involved the molecular structures supporting the reactions involved are of most importance in the design of cell factories for this purpose. The work developed during this thesis was therefore meant to communicate that message through success cases and relevant knowledge, which was hopefully achieved in the eyes of the reader.

## Is there a future for yeast as a cell factory for oleochemicals?

If there is one aspect that should have become transparent during this text, is that by thoroughly understanding the mechanisms that govern metabolism, different research groups have achieved remarkable success in transforming the common baker's yeast into a new cell capable of producing chemicals that the native species was never able to. In regards of lipids, *S. cerevisiae* natively accumulates a very low amount of lipids and it has already been engineered to accumulate more than 50 times these amounts. Moreover, additional progress is being done every day to convert this yeast into an efficient oleaginous species and showcasing the its untamed potential.

To this one should not fall into the idea that the worth of *S. cerevisiae* is in the production of the common C18 and C16 oleochemical species, but as explored before, in its potential to be engineered for production of specific rare species of oleochemicals. The strategies explored during the studies here shown focus on knowledge to increase productivity of FFA and TAGs. However, if this is coupled to product specificity-related engineering, such as engineering of the FAS product specificity (Zhu et al., 2017) or changing the specificity of enzymes in the TAG synthesis pathway (Wei et al., 2017), it is possible to create high levels of tailored products for specialty applications. High levels production of specialty chemicals enables the creation of a cell which is a producer of high value chemicals from biomass. This will in a first step allow for economic feasibility of the technology through simple fermentation of sugars. In a later stage, these cell factories can compose the most-important high-value production part of a biorefinery and take the central pillar that allows for the setup of a biorefinery concept that creates both market value and sustainable solutions to materials and energy.



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